Genetic Analysis of Fluorescent Pigment Production in *Pseudomonas syringae* pv. *syringae*

By JOYCE E. LOPER,† CINDY S. ORSER,
NICKOLAS J. PANOPOULOS AND MILTON N. SCHROTH*
Department of Plant Pathology, University of California, Berkeley, CA 94720, USA

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Genes involved in the biosynthesis of a fluorescent pigment by *Pseudomonas syringae* pv. *syringae* (*P. s. syringae*) JL2000 were investigated. A genomic library of this strain was constructed using the broad host range cosmid vector, pLAFR1. Nonfluorescent (Flu⁻) mutants of JL2000, defective in the biosynthesis of a fluorescent pigment, were obtained after ethyl methanesulphonate mutagenesis. Individual recombinant plasmids from the genomic library were introduced into Flu⁻ mutants. Of a total of 146 Flu⁻ mutants, 36 were restored to fluorescence following matings with individual recombinant colonies in the genomic library. Four separate fluorescence restoration groups, each comprised of 5 to 11 Flu⁻ mutants restored to fluorescence by one of four structurally distinct recombinant plasmids, were identified. Whereas the 36 Flu⁻ mutant strains differed in their abilities to grow on an iron-deficient medium, wild-type *P. s. syringae* strain JL2000 and all Flu⁺ transconjugants from these 36 crosses grew on an iron-deficient medium. These results indicate that at least four genes or gene clusters are involved in the production of a fluorescent pigment of *P. s. syringae* strain JL2000. These genes, with few exceptions, also control the ability of strain JL 2000 to grow under iron-limiting conditions.

INTRODUCTION

Many strains of fluorescent pseudomonads are important agriculturally as soil saprophytes, foliar epiphytes, or phytopathogens. Some of these bacteria colonize the roots of agricultural plants and may be considered beneficial, deleterious, or neutral to plant growth (Schroth & Hancock, 1981, 1982). A number of beneficial strains taxonomically classified in the *Pseudomonas fluorescens*–*putida* group have been used as seed inoculants of agricultural plants to increase plant growth (Suslow, 1982; Burr & Caesar, 1984), or to control biologically one or more soil-borne phytopathogens (Howell & Stipanovic, 1979, 1980; Scher & Baker, 1982; Weller & Cook, 1983). Other strains, classified as pathovars of *Pseudomonas syringae* van Hall, are bacterial epiphytes prevalent on leaf surfaces, with the capability of inciting disease and frost injury on a wide variety of host plants (Schroth *et al.*, 1981).

The singular characteristic common to all strains of fluorescent pseudomonads is the production of an extracellular, water-soluble, yellow-green pigment which fluoresces under UV irradiation. The fluorescent pigment produced by *P. fluorescens* Migula functions as a siderophore, as characterized by its synthesis only under iron-limiting conditions (Lenhoff, 1963), specific and high affinity for Fe³⁺ (Meyer & Abdallah, 1978), and role in transport of Fe³⁺ into the bacterial cell (Meyer & Hornsperger, 1978). The siderophores or related substances produced by *P. fluorescens* include both fluorescent pigments: pseudobactin (Teintze

† Present address: Chevron Chemical Co., 940 Hensley St, Richmond, CA 94804, USA.

Abbreviations: KBM, King's Medium B (King *et al.*, 1954); EDDA, ethylenediaminedi(o-hydroxyphenylacetic acid).

et al., 1981), pyoverdine (Meyer & Abdallah, 1978; Meyer & Hornsperger, 1978; Philson & Llinas, 1982*a, b*) and compound S (McCracken & Swinburne, 1979); and nonfluorescent compounds: ferribactin (Maurer *et al.*, 1968), pseudobactin A (Teintze & Leong, 1981), and unidentified purple compounds (Philson & Llinas, 1982*a*).

The crystal and molecular structure of ferric pseudobactin, a fluorescent pigment-siderophore from *Pseudomonas fluorescens* strain B10, has been shown by X-ray crystallography to consist of a linear hexapeptide of both L- and D-amino acids attached to a fluorophoric quinoline derivative (Teintze *et al.*, 1981). NMR spectroscopy analysis of the pyoverdine compound produced by *P. fluorescens* ATCC 13525 suggests a striking similarity to the structure of pseudobactin, with a possible discrepancy in that the succinyl diamide chain attached to the quinoline moiety may not be the same for pseudobactin as for pyoverdine (Philson & Llinas, 1982*b*). The fluorescent chromophore of pseudobactin (Teintze *et al.*, 1981) and of pyoverdine (Philson & Llinas, 1982*b*) is analogous to that of the *Azotobacter* pigment (Bulen & Le Comte, 1962; Corbin *et al.*, 1970). The structure of the fluorescent pigment of the phytopathogenic species, *P. syringae*, is unknown, but it has been suggested that at least minor structural differences exist among pigments produced by various species of fluorescent pseudomonads (Meyer & Hornsperger, 1978).

The role of siderophores in the ecology of plant-associated *Pseudomonas* species recently has become an area of active research. *Pseudomonas* strains producing siderophores exhibit *in vitro* antibiosis against indicator bacterial or fungal strains (Kloepper *et al.*, 1980; Hemming *et al.*, 1982; Misaghi *et al.*, 1982) which is influenced by addition of iron to the culture medium. The positive influence on plant growth induced by beneficial strains is considered in part a result of the production of extracellular siderophores which complex the iron in the root environment, making it less available to competing microflora, thus alleviating plant stresses induced by soil-borne phytopathogens (Kloepper *et al.*, 1980; Scher & Baker, 1982).

The suggested involvement of siderophores in plant growth promotion and biological control resulting from seed inoculation with beneficial *Pseudomonas* strains has generated interest in the genetics of siderophore production and regulation in these organisms. This study was initiated to identify genes involved in the production of the fluorescent pigment of *Pseudomonas syringae* pv. *syringae* (hereafter referred to as *P. s. syringae*).

A broad host range cloning vector, pLAFR1, developed by Ditta *et al.* (1980) has been used in cloning experiments with non-enteric bacteria (Friedman *et al.*, 1982) including the pseudomonads (Orser *et al.*, 1983). This vector, a cosmid, is suitable for *in vitro* packaging of recombinant molecules since it contains the cohesive ends site (*cos*) of bacteriophage λ . RK2, the parental plasmid of pLAFR1, is known to replicate in several pathovars of *P. syringae* (Panopoulos *et al.*, 1978). Genomic libraries of *P. s. syringae* strains constructed with this vector proved useful in the genetic analysis of fluorescent pigment biosynthesis in *P. s. syringae* merodiploids.

Preliminary aspects of this work have been reported previously (Loper *et al.*, 1983; Orser *et al.*, 1983).

METHODS

Bacterial strains and plasmids. The bacterial strains, resident plasmids, relevant phenotypic characteristics and origins are described in Table 1. *Pseudomonas s. syringae* 31 is a fluorescent (Flu^+), ice-nucleation active (Ina^+), epiphytic strain originally isolated from a corn leaf surface (Arny *et al.*, 1976). Strain JL2000 (31R1), a spontaneous mutant of strain 31, was selected on the basis of its resistance to rifampicin ($100\ \mu\text{g ml}^{-1}$; Sigma). The broad host range cloning vector, pLAFR1 (Friedman *et al.*, 1982), a cosmid, is a 21.6-kb derivative of plasmid pRK290 (Ditta *et al.*, 1980) which carries the cohesive ends site (*cos*) of bacteriophage λ , has a single *EcoRI* restriction enzyme site suitable for cloning DNA fragments, and confers resistance to tetracycline. The recombinant helper plasmid, pRK2013, carries the conjugal transfer gene system and kanamycin resistance marker of RK2 linked to the ColE1 replicon (Figurski & Helinski, 1979; Ditta *et al.*, 1980).

Isolation of nonfluorescent (Flu^-) mutants of *P. s. syringae*. Mutants of JL 2000 deficient in fluorescent pigment production were derived by chemical mutagenesis with ethyl methanesulphonate (methanesulphonic acid ethyl ester; EMS) to achieve 90% killing of bacterial cells. Strain JL2000 was grown in KBM (King *et al.*, 1954) broth to exponential phase, 5% (v/v) EMS was added, mixed well, and incubated (25 °C) with shaking for 20 min.

Table 1. *Origin and characteristics of bacterial strains*

Strain JL2000 and its derivatives did not contain indigenous plasmids detectable by methods cited in the text.

Strain no.	Relevant genetic and phenotypic markers*		Origin
	Chromosomal	Plasmid	
<i>Escherichia coli</i>			
HB101	F ⁻ <i>hsdS20</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>recA13</i> <i>ara-14 proA2 lacY1 galK2</i> <i>rpsL20</i> (Str ^r) <i>xyl-5 mtl-1</i> <i>supE44</i> λ ⁻	None	N. Panopoulos
JL1101	As above	pLAFR1 (Tet ^r <i>cos tra</i> ⁻ <i>rlx</i> ⁺)	HB101(pLAFR1); S. Long (Friedman <i>et al.</i> , 1982)
JL1102	As above	pRK2013 (Kan ^r <i>tra</i> ⁺)	HB101(pRK2013); S. Long (Ditta <i>et al.</i> , 1980)
JL1010	As above	pSFL10† (Tet ^r <i>cos tra</i> ⁻ <i>rlx</i> ⁺)	See text
JL1011	As above	pSFL11† (Tet ^r <i>cos tra</i> ⁻ <i>rlx</i> ⁺)	See text
JL1012	As above	pSFL12† (Tet ^r <i>cos tra</i> ⁻ <i>rlx</i> ⁺)	See text
JL1014	As above	pSFL14 (Tet ^r <i>cos tra</i> ⁻ <i>rlx</i> ⁺)	See text
<i>Pseudomonas syringae</i> pv. <i>syringae</i>			
JL2000	Flu ⁺ Ina ⁺ Rif ^r Eda ⁺	—‡	31R1; S. Lindow (Army <i>et al.</i> , 1976)
JL2003, JL2023	Flu ⁻ Ina ⁺ Rif ^r Eda ⁻	—‡	EMS mutagenesis of JL2000 (see text)
JL2087, JL2088	Flu ⁻ Ina ⁺ Rif ^r Eda ⁺	—‡	EMS mutagenesis of JL2000 (see text)
JL2140	Flu ⁻ Ina ⁻ Rif ^r Eda ⁻	—‡	31R1-26; S. Lindow (Lindow & Staskawicz, 1981)

* *E. coli* chromosomal markers are according to Bachmann & Low (1980). Abbreviations: Eda⁺, growth on KBM-EDDA (200 µg ml⁻¹); Flu⁺, fluorescent pigment production; Ina⁺, ice nucleation activity. Rif, Kan, Tet, and Str refer to rifampicin, kanamycin, tetracycline and streptomycin. Superscript r or s refers to relative resistance or susceptibility. *cos* = cohesive ends site of phage λ . *tra*⁺ = conjugative plasmid transfer. *rlx*⁺ = relaxation site.

† Prefix registered with the Plasmid Reference Center, Department of Medical Microbiology, Stanford University, Stanford, Calif., USA.

‡ No indigenous plasmids have been detected in strain JL2000 or its derivatives in repeated attempts using standard methods described in text.

Mutagenized cells were washed twice, resuspended in KBM broth, and incubated for 2 h to allow chromosome segregation and phenotypic lag (Miller, 1972). Segregated cells were then plated on KBM agar at cell densities of 30–50 c.f.u. per plate. After 2 d incubation at 25 °C, Flu⁻ mutants were detected by observation under UV irradiation (366 nm).

Construction of genomic library. A genomic library, designated *PssLIB2*, was constructed by cloning total DNA from *P. s. syringae* strain JL2000 in *Escherichia coli* HB101 (Orser *et al.*, 1983) using the methods of Friedman *et al.* (1982). The DNA was extracted following cell lysis in a Tris/sucrose/EDTA/lysozyme mixture [50 mM-Tris buffer, pH 7.8, 25% (w/v) sucrose, 5 mM-EDTA, 1 mg lysozyme ml⁻¹], banding twice in caesium chloride/ethidium bromide density gradients using a Beckman vertical rotor at 45000 r.p.m. for 20 h, dialysis of the DNA containing fractions in Tris/EDTA buffer (10 mM-Tris, pH 8.0, 1 mM-EDTA) and sequential precipitation of the DNA with 1 vol. isopropanol and 2 vol. 95% (v/v) ethanol. The final pellet was resuspended in 10 mM-Tris, 1 mM-EDTA, pH 8.0, and subjected to partial digestion with *EcoRI* restriction endonuclease (0.3 units per µg DNA; Bethesda Research Laboratories (BRL), Rockville, Md., USA). The reaction was stopped by heating at 65 °C for 4 min. The DNA was fractionated by sucrose gradient centrifugation (5–25% sucrose, 20000 r.p.m., 18 h) and 20 µl portions of the fractions were electrophoresed on ethidium bromide/agarose gels (0.5 µg ethidium bromide ml⁻¹, 0.7% agarose, 40 mM-Tris, 20 mM-acetic acid, 2 mM-Na₂EDTA buffer, pH 8.1). The fractions rich in 18–25 kb fragments were pooled, precipitated with ethanol and resuspended in sterile distilled water to a final concentration of 1 µg ml⁻¹. DNA of the cosmid vector pLAFR1 was similarly purified and digested to completion with *EcoRI*.

One μl of this preparation and 4 μl of genomic DNA fragments (1 and 4 μg , respectively) were mixed and sequentially incubated at 65 °C for 2 min, at 42 °C for 30 min, and at room temperature for 2 h. Ligation was performed in the presence of a total of 5 units of T4 DNA ligase (BRL) and 1 mM-ATP at 12 °C overnight. *In vitro* packaging extracts were prepared from strains BHB2690 and BHB2688 as described in Maniatis *et al.* (1982). For packaging, 30 μl of sonic extract and 20 μl of freeze-thaw extract, 2 μl of 1 mM-ATP and 5 μl of ligated DNA were mixed and incubated at room temperature for 1 h. One ml of a dilution buffer (10 mM-MgCl₂, 10 mM-Tris buffer, pH 7.6) was added and the samples were stored at 4 °C. For transduction, 0.1 ml of the above phage stock was mixed with 0.5 ml of *E. coli* HB101 cells grown to mid-exponential phase (10^7 – 10^8 cells ml⁻¹) in Luria broth (Miller, 1972) supplemented with 0.4% maltose and incubated for 1 h at 37 °C. Two ml Luria broth was then added and the cells were allowed to grow for 1.5–2 h at 37 °C. Transductants (hereafter referred to as *E. coli* clones) harbouring recombinant plasmids were selected on Luria agar supplemented with 15 μg tetracycline ml⁻¹.

DNA analysis. Rapid isolation of plasmid DNA was done by an alkaline extraction procedure as described by Birnboim & Doly (1979) or by the boiling lysis procedure described by Maniatis *et al.* (1982). Restrictionendonuclease digestions and agarose gel electrophoresis were performed as described by Maniatis *et al.* (1982).

Transfer of recombinant plasmids to *Flu*⁻ mutants. The conjugative plasmid, pRK2013, which mobilizes the vector, pLAFR1, was used as a helper element to transfer the recombinant plasmids of *PssLIB2* to recipient *Flu*⁻ mutants in triparental matings (Ditta *et al.*, 1980). These matings were carried out as follows. Exponential phase cultures of the entire *PssLIB2* library (*en masse* matings) or of individual *E. coli* clones, JL1102, and individual *Flu*⁻ mutants were mixed to a concentration of approximately 10^8 , 10^8 and 3×10^9 c.f.u. ml⁻¹ respectively, and placed on 0.2 μm membrane filters (Millipore). The filters were incubated overnight on KBM agar before the cells were suspended in 100 mM-MgSO₄, serially diluted and plated on KBM agar containing rifampicin (Rif, 100 μg ml⁻¹) and tetracycline (Tet, 15 μg ml⁻¹) to select for Tet^r, Rif^r transconjugants. These transconjugants were screened for fluorescent pigment production after incubation (4 d) at 25 °C by illuminating the selection plates with UV (366 nm).

Alternatively, each of 718 *E. coli* clones of *PssLIB2* were mixed with JL1102 and individual *Flu*⁻ mutants on KBM in separate wells of microtitre plates. The microtitre plates were incubated for 18–24 h at 25 °C. Rif^r, Tet^r transconjugants of *P. s. syringae* were obtained by transferring cells from the microtitre plates to selective medium with a 49-prong replicator. Restoration of fluorescence was scored as described above.

Fluorescent *P. s. syringae* transconjugant colonies were purified and used as donors in individual triparental matings as above but with HB101 as a recipient. Exponential phase cultures of individual *P. s. syringae* transconjugant strains, JL1102, and HB101 were mixed to a concentration of approximately 10^9 , 10^8 , and 10^8 c.f.u. ml⁻¹ respectively, placed on 0.2 μm membrane filters, and incubated overnight on Luria agar plates. Tet^r, Str^r transconjugants of *E. coli* HB101 were selected on Luria medium containing streptomycin (Str, 15 μg ml⁻¹) and tetracycline (15 μg ml⁻¹). To confirm the ability of each recombinant cosmid to restore fluorescence to the original *Flu*⁻ mutant after retransfer to *E. coli*, purified Tet^r, Str^r transconjugants from the above crosses were used as donors in triparental matings with *Flu*⁻ *P. s. syringae* recipients.

The fluorescence of some *P. s. syringae* transconjugants was unstable upon subculture. The transconjugant strains discussed in this paper are those which expressed the fluorescence phenotype stably through several sequential single-colony transfers on selective medium (KBM with rifampicin and tetracycline).

Conditions of iron-limited growth. KBM agar was supplemented with EDDA (200 μg ml⁻¹; Sigma), resulting in a medium (KBM-EDDA) with low levels of available iron. The EDDA solution was prepared as described by Ong *et al.* (1979) after removing iron as described by Rogers (1973). KBM agar supplemented with EDDA was allowed to stand for at least 24 h at 4 °C prior to use to allow slow chelation of iron. The specificity of EDDA chelation for the ferric ion is discussed in Archibald & De Voe (1980). Bacterial suspensions were adjusted to a uniform density of 0.1 OD₆₄₀ unit and streaked with a calibrated 1 μl loop on KBM-EDDA medium. Only four suspensions were streaked on each plate to negate the influence of cross-feeding. Iron-limited growth inhibition is reversed by addition of a freshly prepared, filter-sterilized solution of 10^{-2} M-FeCl₃ to the KBM-EDDA medium to a final concentration of 10^{-4} M-FeCl₃.

RESULTS

EMS mutagenesis

Nonfluorescent mutants of *P. s. syringae* strain JL2000 arose at a frequency of 0.1–0.5% of cells plated following EMS mutagenesis. No spontaneous *Flu*⁻ mutants of JL2000 were observed in cultures not subjected to EMS mutagenesis. A total of 146 *Flu*⁻ mutants were selected for use as recipients in triparental matings with *PssLIB2*. Many of these mutants failed to grow on an iron-deficient medium (KBM-EDDA), a phenotype designated here as *Eda*⁻ (see Table 1). Reversion of the mutants to *Flu*⁺ and *Eda*⁺ was never observed. Since no direct

selection for Flu⁺ or Eda⁺ is available (growth on KBM-EDDA is dependent on plating density, presumably due to cross-feeding among cells), the exact reversion frequency could not be determined. Nevertheless, the fact that only certain recombinant plasmids were able to restore fluorescence to a given group of Flu⁻ mutants while others consistently failed to do so (see below) provides an internal control which obviates the need for more accurate reversion frequency estimates for the interpretation of data presented below.

Fluorescence restoration experiments

En masse matings of *PssLIB2* with one Flu⁻ mutant, JL2140, yielded Tet^r transconjugants at a frequency of approximately 1.5×10^{-4} per recipient. Triparental matings of JL2140 with 718 individual *E. coli* clones comprising the genomic library resulted in the identification of two recombinant plasmids producing Flu⁺ transconjugants. *EcoRI* restriction endonuclease digestion patterns of the recombinant plasmids isolated from both *E. coli* donors were identical indicating that these donors were probably duplicate clones stored separately in microtitre plates. The plasmid common to both clones was designated pSFL10 and the HB101 clone harbouring this plasmid was named JL1010.

Plasmid pSFL10 was subsequently transferred by individual triparental matings of JL1010 with 145 additional Flu⁻ mutants of *P. s. syringae* strain JL2000. Tet^r transconjugants were selected in every case at frequencies of 9×10^{-5} to 1×10^{-3} , but fluorescence was restored in only 10 Flu⁻ mutants (including JL2140) upon introduction of pSFL10.

Seven Flu⁻ mutants which were not restored to Flu⁺ upon introduction of pSFL10 were individually mated with JL1102 and each of the 718 members of the DNA genomic library. Three *E. coli* clones, JL1011, JL1012 and JL1014, were identified which gave Tet^r, Flu⁺ transconjugants upon transfer to one or more of the seven Flu⁻ mutant strains. Each of these *E. coli* clones was then mated separately with each of the 146 Flu⁻ mutants. In every case, Tet^r transconjugants could be selected at similar frequencies as in previous crosses. The numbers of Flu⁻ mutants yielding Flu⁺ transconjugants in these crosses were 10, 11 and 5 for JL1011, JL1012, and JL1014 respectively (Table 2). None of the mutants were restored to Flu⁺ by mating with more than one of the four *E. coli* clones.

These experiments define four fluorescence restoration groups, designated as groups 10, 11, 12 and 14 (Table 2) and defined for the purposes of this study as an *E. coli* strain harbouring a recombinant plasmid and a group of 5 to 11 Flu⁻ mutants of *P. s. syringae* strain JL2000 for which fluorescence was restored by that clone. One or more representative mutants from each fluorescence restoration group are given in Table 2. Whereas a total of 36 Flu⁻ mutants were restored to Flu⁺ in these experiments, the remaining 110 mutants were not restored by any of the

Table 2. *Fluorescence restoration groups and iron-limited growth of P. s. syringae strains*

Individual triparental matings of the four *E. coli* plasmid donors with each of the 146 nonfluorescent mutants yielded Tet^r transconjugants in every case. The number of mutants yielding Flu⁺, Tet^r transconjugants in matings with *E. coli* donors is given in column 4. All nonfluorescent mutants and fluorescent transconjugants grew on KBM and on KBM-EDDA supplemented with 10^{-4} M-FeCl₃. All fluorescent transconjugants grew on KBM-EDDA.

Fluorescence restoration group	<i>E. coli</i> donor strain	Recombinant plasmid	No. of Flu ⁻ mutants restored to Flu ⁺	Growth of representative nonfluorescent mutants on KBM-EDDA	
				Strain	Growth
10	JL1010	pSFL10	10	JL2140	—
11	JL1011	pSFL11	10	JL2003	—
12	JL1012	pSFL12	11	JL2023	—
				JL2088	+*
14	JL1014	pSFL14	5	JL2087	+†

* Strain JL2088 grew on KBM-EDDA plates, but exhibited no visible fluorescence on KBM or KBM-EDDA.

† Strain JL2087 grew and exhibited weak fluorescence on KBM-EDDA but exhibited no visible fluorescence on KBM.

four plasmids. We presume that these mutants carried mutation(s) in genes which had not been cloned or had been inactivated by endonuclease digestion. It is also possible that, given the severe mutagenic treatment employed, these mutants carried multiple mutations that were not clustered within the DNA fragments carried by these plasmids.

Structural analysis of recombinant plasmids

Digestion of DNA of the four recombinant plasmids (pSFL10, pSFL11, pSFL12, pSFL14) isolated from the original *E. coli* clones with the restriction endonuclease *Eco*RI indicated that the four recombinant plasmids were structurally distinct (Fig. 1).

Repeated attempts to demonstrate the presence of a plasmid with an *Eco*RI restriction pattern having a 21.6 kb fragment corresponding to pLAFR1 in any of the *P. s. syringae* transconjugants of Table 2 were unsuccessful. That these transconjugants had indeed acquired recombinant plasmids following conjugation was determined indirectly as follows. Four individual Flu⁺, Tet^r transconjugants from each of the four complementation groups were utilized as donors in triparental matings with JL1102 and *E. coli* HB101 as the recipient. Tet^r, Str^r transconjugants were selected at a frequency of approximately 10⁻⁵ per recipient. Several

Fig. 1. Agarose gel electrophoresis of *Eco*RI-generated fragments of recombinant plasmids: (a) pSFL14, (b) pSFL12, (c) pSFL11, (d) pSFL10 and (e) pLAFR1. Purified plasmid DNA was cut with *Eco*RI and fractionated on a 0.7% (w/v) agarose gel in Tris/acetate buffer (Maniatis *et al.*, 1982) at 120 V, 60 mA for 4 h. Lane (f) is phage λ cut with the restriction endonuclease *Hind*III. Numbers represent DNA sizes in kb.

transconjugants from each cross were analysed for their plasmid content following *EcoRI* digestion. In most cases, the *EcoRI* restriction fragment patterns observed were composites of the two fragments of pRK2013 and those of the recombinant plasmid (pSFL10, pSFL11, pSFL12, or pSFL14) isolated from the original recombinant clones. A small percentage of the Tet^r, Str^r transconjugants contained fragments corresponding to those of the original recombinant cosmid but lacked the pRK2013 fragments. The presence of the pRK2013 fragments always correlated with resistance to kanamycin, conferred by this plasmid. These recombinant plasmids were functionally intact after transfer to *E. coli* since reintroduction to mutants within their respective fluorescence restoration groups was accompanied by restoration of the Flu⁺ phenotype.

Expression of iron-limited growth phenotype

The fluorescent, wild-type *P. s. syringae* strain JL2000 and all Flu⁺ transconjugants grew on the iron-limiting medium, KBM-EDDA, while Flu⁻ mutants of different fluorescence restoration groups differed in their growth (Table 2). Addition of 10⁻⁴ M-FeCl₃ to KBM-EDDA reversed the growth inhibition in every case, presumably because of increased iron availability. All Flu⁻ mutants of groups 10 and 11 were unable to grow on KBM-EDDA, as exemplified by strains JL2140 and JL2003, respectively. In contrast, all Flu⁻ mutants of complementation group 14 grew on KBM-EDDA, as exemplified by strain JL2087. Furthermore, all mutants of group 14, while nonfluorescent on KBM, did fluoresce slightly on KBM-EDDA. Flu⁻ mutants of group 12 fell into two groups on the basis of their ability to grow on KBM-EDDA. Seven mutants of group 12 (subgroup 12a) exhibited growth patterns similar to the Flu⁻ mutants of groups 10 and 11. The behaviour of these mutants is exemplified by strain JL2023, which was unable to grow on KBM-EDDA. Four Flu⁻ mutants of group 12 (subgroup 12b) did grow on KBM-EDDA, as exemplified by strain JL2088. However, these mutants differed from those of fluorescence restoration group 14 in that they were nonfluorescent on both KBM and KBM-EDDA.

DISCUSSION

At least four distinct genes or gene clusters are involved in fluorescent pigment biosynthesis of *P. s. syringae*. Four recombinant plasmids, containing DNA fragments involved in some aspect of fluorescent pigment production, were distinct structurally, in their *EcoRI* restriction patterns, and functionally, in their lack of overlap in fluorescence restoration. Nonfluorescent mutants of fluorescence restoration group 12 fell into two groups based on their growth under iron-limiting conditions. Two or more genes involved in distinct aspects of fluorescent siderophore production may be present on the DNA fragment(s) cloned on the recombinant plasmid pSFL12, which restored these mutants with respect to fluorescence. The lack of any common *EcoRI* fragments among the four recombinant cosmids analysed suggests that the genes for fluorescent pigment (siderophore) biosynthesis in *P. s. syringae* are located in at least four distinct gene clusters. In contrast, genes for biosynthesis of the high-affinity siderophores, aerobactin and enterobactin, in *E. coli* are in single gene clusters within a 16.3 kb fragment (aerobactin, Bindereif & Neilands, 1983) or a 26 kb fragment (enterobactin, Laird & Young, 1980; Laird *et al.*, 1980). Generally, lack of clustering of functionally related genes is more common in *Pseudomonas* spp. than in the enteric bacteria (Holloway *et al.*, 1979).

Studies analysing the structure of fluorescent siderophores of *P. fluorescens* have identified both hydroxamate groups involved in iron chelation and a fluorescent chromophore on the same molecule (Teintze *et al.*, 1981; Philson & Llinas, 1982*b*). Mutants deficient in the production of this fluorescent siderophore are expected to be deficient both in fluorescence and in their ability to grow under iron-limiting conditions. Flu⁻ mutants of groups 10 and 11 and of sub-group 12a exhibited these pleiotropic functions, as expected. However, other mutants, either completely or partially deficient in fluorescence, grew but differed in their fluorescence under iron-limiting conditions. Flu⁻ mutants of fluorescence restoration group 14 were slightly fluorescent on iron-limiting medium whereas those of sub-group 12b were not. One can envisage that mutants of

group 14 are altered in their iron regulation. That is, although the fluorescent siderophore is not produced by these mutants on KBM as in the wild-type strain, production does occur under more iron-limiting conditions. Nonpleiotropic mutants of sub-group 12b may produce reduced levels of the fluorescent siderophore that are sufficient for iron-limited growth but insufficient for visible fluorescence. Alternatively, these mutants may be altered only in the synthesis or attachment of the fluorescent chromophore of the siderophore, retaining at least reduced iron-chelating and transport functions of this molecule. Ferribactin and pseudobactin A, siderophores structurally related to pyoverdine and pseudobactin but lacking the functional fluorescent chromophore of these compounds, have been isolated from *P. fluorescens* (Teintze & Leong, 1981; Philson & Llinas, 1982*a,b*). The chemical structures of pseudobactin and pseudobactin A, siderophores isolated from culture filtrates of *P. fluorescens* strain B10, differ only in the absence of a double bond in the fluorescent chromophore of pseudobactin. The siderophore produced by nonpleiotropic mutants of sub-group 12b may be analogous to ferribactin or pseudobactin A, exhibiting at least reduced iron-chelating and transport functions but not fluorescence.

A large number of Flu⁻ mutants could not be restored to Flu⁺ by the four recombinant plasmids identified in this study. The existence of additional genes or gene clusters involved in fluorescent pigment biosynthesis of *P. s. syringae* is not definitive at this time.

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