

Lack of Evidence for In Situ Fluorescent Pigment Production by *Pseudomonas syringae* pv. *syringae* on Bean Leaf Surfaces

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ABSTRACT

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The fluorescent siderophore of *Pseudomonas syringae* pv. *syringae* (*P. s. pv. syringae*) is a chromophore effective in protecting the producing cells from exposure to ultraviolet light (UV) ($\lambda = 254$) in culture. *P. s. pv. syringae* strain B728a, which causes brown spot disease of bean, produces a fluorescent pigment and grows on the iron-deficient King's B medium (KBM) supplemented with ethylenediamine(*o*-hydroxyphenylacetic acid) (EDDA). Strain I-1, a nonfluorescent (Flu-) derivative of B728a obtained after ethyl methanesulfonate mutagenesis, did not grow on the KBM-EDDA medium. Strain I-1 was more sensitive than the parental strain B728a to UV irradiation in culture. LD₅₀ values, calculated for UV

killing curves, were 142.4 ± 1.4 and 100.8 ± 1.8 erg/mm² for strains B728a and I-1, respectively. Growth rates, stationary epiphytic population sizes, number of brown spot lesions, and UV sensitivities of B728a and I-1 did not differ significantly on leaf surfaces of greenhouse-grown bean plants. No consistent differences were observed between four parental and four Flu- derivative strains with respect to their population sizes on bean leaf surfaces over a 7-day period on field-grown bean plants. These indirect studies provide no evidence of in situ fluorescent siderophore production by *P. s. pv. syringae* or of its contribution to the growth, survival, or pathogenicity of *P. s. pv. syringae* on bean leaf surfaces.

Many strains of fluorescent pseudomonads are important agriculturally as soil or rhizosphere saprophytes, foliar epiphytes, or phytopathogens. Pathovars of *Pseudomonas syringae* van Hall with the capability of inciting disease or frost injury on a wide variety of host plants are bacterial epiphytes prevalent on leaf surfaces (30). The singular characteristic common to all strains of fluorescent pseudomonads is the production of an extracellular, water-soluble, yellow-green pigment that fluoresces under ultraviolet (UV) irradiation. The fluorescent pigments produced by *P. fluorescens* Migula and *P. syringae* function as siderophores, as characterized by their synthesis only under iron-limiting conditions (13), specific and high affinity for the ferric ion (20,33,35), and role in transport of Fe³⁺ into the bacterial cell (17,21).

The speculative roles for the fluorescent pigment in the phylloplane ecology of phytopathogenic strains of *P. syringae* include the following: 1) If the chemical environment of the leaf surface is iron-deficient, the iron-chelating and iron-transport properties of this pigment may determine the growth and virulence of the producing bacterium. 2) The pigment is known to function as a UV chromophore (35) and may protect producing bacterial cells on leaf surfaces from incident UV or visible irradiation. A yellow pigment produced by *Erwinia herbicola* is ineffective in protecting producing cells against UV irradiation but may be involved in photoreactivation, perhaps influencing the survival of pigmented *E. herbicola* cells on aerial plant surfaces (5). 3) The fluorescent siderophores of *P. syringae* and other fluorescent pseudomonads are involved in their antagonisms against indicator bacteria and fungi in culture, (7,15,23; S. E. Lindow, *unpublished*) and may also contribute to their antagonistic characteristics on leaf surfaces. The fluorescent pigments of several *P. fluorescens* strains are involved in antagonism of soilborne fungi in the rhizosphere (16,28,29). This study was initiated to determine if fluorescent pigment production of the brown spot pathogen of bean, *P. syringae* pv. *syringae* (*P. s. pv. syringae*), influences its growth, survival, or virulence on bean (*Phaseolus vulgaris* L.) leaf surfaces.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. *P. s. pv. syringae* strains were routinely grown on nutrient agar or in nutrient broth (Difco Laboratories, Detroit, MI) supplemented with 1% (w/v) glycerol (NGA or NGB, respectively). Fluorescence of cultures was observed under UV light ($\lambda = 366$ nm) on King's medium B (KBM) (9) after 24–48 hr of incubation at 25 C. Prototrophy was evaluated by observation of growth on a minimal medium containing (grams per liter) K₂HPO₄, 3; NaH₂PO₄, 1; NH₄Cl, 1; MgSO₄·7H₂O, 0.4; glycerol, 15; and agar, 15. A defined medium (SM) used to optimize fluorescence contained (grams per liter): (NH₄)₂SO₄, 1.32; MgSO₄·7H₂O, 0.25; glutamine, 1.00; glycerol, 10.00; HEPES buffer, 0.05 M; and potassium phosphate buffer, 0.005 M, pH 7.5. Population doubling times (t_d) were determined from changes in A_{640nm} with time of cultures grown at 30 C in NGB or in SM supplemented with various concentrations of FeCl₃. The antibiotics rifampicin and cycloheximide (Sigma) were each used when specified at 100 µg/ml.

Four strains of *P. s. pv. syringae* that cause brown spot of bean and express ice nucleation activity were isolated from bean leaves in Wisconsin by J. Lindemann. Spontaneous mutants of these strains with resistance to rifampicin were obtained by spreading about 10⁹ cells per plate of KBM supplemented with rifampicin. After 3 days of incubation at 30 C, single colonies from antibiotic selection plates were isolated. Rifampicin-resistant derivatives were fluorescent and prototrophic, had the same growth rates as their parental strains in NGB, and showed stable resistance to rifampicin after more than 30 generations of growth without selection. Rifampicin-resistant derivatives of the four *P. s. pv. syringae* field isolates were designated B728a, B728b, B733, and J944.

Inoculum of bacteria for greenhouse or field application was harvested after 3 days of growth at 20 C from plates of KBM containing rifampicin (KBMR).

Isolation of nonfluorescent (Flu-) mutants of *P. s. pv. syringae*. Mutants deficient in fluorescent pigment production were derived by chemical mutagenesis with ethyl methanesulfonate (EMS) (Sigma). Cultures growing in KBM broth at the exponential phase

were treated with EMS (5%, v/v), mixed well, and incubated (25 C) with shaking for 20 min to achieve 90% killing of bacterial cells. Mutagenized cells were washed twice, resuspended in KBM broth, and incubated for 2 hr to allow chromosome segregation and phenotypic expression (22). Segregated cells were then plated on KBM agar at cell densities of 30–50 colony-forming units (cfu) per plate. After 2 days of incubation at 25 C, Flu⁻ mutants were detected by observation under UV light ($\lambda = 366$ nm). Flu⁻ mutants of B728a, B728b, B733, and J944 were designated I-1, O-1, P-1, and A-2, respectively.

Conditions of iron-limited growth. KBM was supplemented with 200 μ g/ml ethylenediaminedi(*o*-hydroxyphenylacetic acid) (EDDA) (Sigma) resulting in a medium (KBM-EDDA) with low levels of available iron. The EDDA was prepared as described by Ong et al (25) after removing iron as described by Rogers (27). KBM-EDDA was kept at 4 C for 24 hr before use to allow slow chelation of iron. Bacterial suspensions were adjusted to a uniform density of 0.2 $A_{640\text{nm}}$ units (about 2×10^8 cfu/ml) and streaked with a calibrated 1- μ l loop on KBM-EDDA medium. Only four suspensions were streaked on each plate to minimize the influence of cross-feeding of nonfluorescent mutant strains.

Measurement of UV sensitivity in culture. Sensitivity was determined on agar and in broth media. For agar medium studies, single colonies of *P. s. pv. syringae* B728a and of its Flu⁻ derivative, I-1, grown on KBM were individually suspended in sterile distilled water to a uniform density of 0.1 $A_{640\text{nm}}$ units (about 1×10^8 cfu/ml). Suspensions were diluted 100-fold, and 10- μ l aliquots containing about 1×10^4 cfu were spotted on membrane filters (0.22- μ m pore size, 13 mm diameter, Millipore Corp., Bedford, MA) on the surface of SM agar plates. Plates were incubated at 20 C for 14 hr before exposure to a UV source lamp ($\lambda = 254$ nm, 3 erg/mm²/sec). Duplicate filters for each strain were exposed to UV for 5- to 25-sec intervals. Filters were then aseptically transferred from the agar surface to tubes containing 2 ml of 0.1 M MgSO₄. Numbers of viable cells were estimated by dilution plating on KBM.

For broth culture studies, *P. s. pv. syringae* B728a and its Flu⁻ derivative, I-1, were grown in SM broth for 48 hr at 25 C with shaking (150 rpm), at which time a yellow-green pigment was visible in the culture medium of strain B728a. Cells were removed from the culture medium by centrifugation, washed, and resuspended in sterile water to a uniform density of 0.1 $A_{640\text{nm}}$ units (about 1×10^8 cfu/ml). Culture supernatants were passed through a membrane filter (0.22- μ m pore size). Cell suspensions were diluted 1:100 in culture filtrates. One milliliter of the cell-filtrate suspension was placed in a watch glass (5 cm diameter). Watch glasses were placed on a rotary shaker (100 rpm) beneath a UV source lamp ($\lambda = 254$ nm) at a distance to receive a UV exposure of 10.3 erg/mm²/sec. Ten-microliter aliquots of each of the cell-filtrate suspensions were removed at 10-sec intervals, and numbers of viable cells were estimated by dilution plating on KBM.

Experiments to determine UV sensitivities of cells in culture and on leaf surfaces (described below) were done under darkroom safelamps equipped with red filters to minimize the effects of photoreactivation. The LD₅₀ values of parental and Flu⁻ derivatives were calculated from the slope of the line relating the natural log of the initial number of cells divided by the number of cells remaining after exposure to a given UV dosage.

Greenhouse experiments. Four bean (cultivar Bush Blue Lake) seeds were planted in 4-in. pots containing acid-washed sand and watered with distilled water until emergence. After emergence, plants were watered exclusively with 25% Steinberg's solution (31) with or without added FeCl₃. Iron chlorosis was observed on the trifoliolate leaves of those plants deprived of iron about 14 days after emergence. Once iron chlorosis was observed, plants from both the healthy and iron-chlorotic groups were inoculated by atomizing an aqueous suspension of *P. s. pv. syringae* (about 10^5 cfu/ml) uniformly over the foliage. Plants were misted with distilled water in a mist chamber maintained at about 24 C to allow bacterial growth on leaf surfaces. Ten individual trifoliolate leaves were harvested for use in leaf-surface UV kill curves (described below).

Four replicates, each containing 10 trifoliolate leaves, were included in estimates of epiphytic bacterial population sizes.

Iron content of leaves was measured from perchloric acid digests of dried, ground leaves using an atomic absorption spectrometer as described by Wallihan (36). Four replicates, each containing 40 leaves, were analyzed for iron content.

Field experiments. Field research was done in 1982 on plots in Berkeley, CA. Plots were set up in a randomized complete block design with four replicates. Bean plants (cultivar Bush Blue Lake) were grown to the trifoliolate leaf stage before inoculation. Inoculum was applied by spraying an aqueous suspension of about 5×10^7 cells per milliliter uniformly over plant foliage. At each harvesting time, 10 trifoliolate leaves were bulked as a sample from each replicate. These leaves were weighed (ranging from 4 to 9 g per sample), and epiphytic population size was determined as described below.

Measurement of UV sensitivity on leaf surfaces. *P. s. pv. syringae* was grown on bean leaf surfaces in a mist chamber in the greenhouse for 2–3 days after inoculation. Misting was discontinued 12 hr before UV irradiation to minimize the shielding effect of water droplets on leaf surfaces. Ten individual trifoliolate leaves were evaluated at each UV exposure level. Each leaf was weighed and exposed to UV radiation (10.3 erg/mm²/sec, $\lambda = 254$ nm) on both surfaces for 10–80 sec to receive leaf surface exposure levels of up to 824 erg/mm². Viable leaf surface populations were then evaluated from individual leaves by dilution plating of leaf washings as described below. LD₅₀ values were calculated as described for UV studies in culture.

Estimation of bacterial population sizes on leaf surfaces. Individual leaves (leaf surface UV kill curves) or bulked leaf samples were placed in 100 ml of washing buffer [0.1 M potassium phosphate buffer, 0.1% (w/v) bacto-peptone (Difco), pH 7.0] in a 250-ml Erlenmeyer flask. Flasks were placed in an ultrasonic cleaning bath for 4 min to remove bacterial cells from leaves. Dilutions of leaf washings were plated on KBMR containing cycloheximide (KBCR). Phylloplane population size was estimated from plate counts made after 3 days of incubation at 24 C.

Data analysis. Statistical analysis was accomplished by the univariate and general linear models procedures provided by Statistical Analysis Systems (SAS release 79.6, SAS Institute, Inc., Cary, NC). Because of the lognormality of these and other data sets (8) describing phylloplane bacterial populations, the logarithmic transformation was applied to all values before analysis by the general linear models procedures. The logarithmic transformation was also applied to counts of brown spot lesions before analysis to achieve normality and homogeneity of variances.

RESULTS

Characterization of Flu⁻ mutants. Mutants of *P. s. pv. syringae*, deficient in fluorescent pigment production, were obtained after EMS mutagenesis. These mutants, which no longer fluoresced under UV irradiation, were also unable to grow on KBM-EDDA unless the medium was supplemented with 10^{-4} M FeCl₃. These results indicate that the Flu⁻ mutants exhibit the nonfluorescence and iron-dependent growth phenotype characteristic of strains deficient in fluorescent siderophore production.

P. s. pv. syringae B728a grew on KBM-EDDA, whereas its Flu⁻ derivative, I-1, did not. However, no differences were observed with respect to the influence of FeCl₃ concentration on the growth rate of these strains in SM medium. Both B728a and I-1 grew at a slower rate in unsupplemented SM medium than in SM medium to which 10^{-4} or 10^{-5} M FeCl₃ was added. However, the growth rate of I-1 in unsupplemented SM medium was at least as great as that of B728a (average population doubling times of 3.2 and 4.0 for I-1 and B728a, respectively, in three independent experiments). Furthermore, the percentage decrease in population doubling time with the addition of 10^{-4} M FeCl₃ was similar for both strains (15.3 and 19.5% for I-1 and B728a, respectively). Fluorescent pigment was observed in culture supernatants of B728a grown in unsupplemented SM medium but not in SM containing 10^{-4} M

added FeCl_3 . No fluorescence was observed in culture supernatants of the Flu- derivative, I-1.

UV sensitivity in culture. *P. s. pv. syringae* B728a and its Flu- derivative, I-1, were compared with respect to UV sensitivity in culture. Killing of cells by UV irradiation occurred with first-order kinetics, in which the number of cells lost at a given time was proportional to the number of cells remaining. On membrane filter surfaces, strain B728a was less sensitive to UV than the Flu- strain, I-1. LD_{50} s of B728a and I-1 were 17.8 ± 0.4 and 11.2 ± 0.4 erg/mm^2 , respectively. The cell densities of the parental and Flu- derivative strains were similar on nonirradiated filters, 7.6×10^6 and 7.1×10^6 cfu/filter , respectively. It is therefore unlikely that shielding by neighboring cells differed substantially between the strains or accounted for the observed differences in UV sensitivity. In broth culture, survival of UV exposure was a function of the culture filtrate in which the cells were suspended rather than of the cells themselves (Fig. 1). LD_{50} s of the parental and the Flu- derivative strains were significantly greater when suspended in the culture filtrate of the parental strain in which the extracellular pigment was visible than when suspended in the culture filtrate of the Flu- derivative strain (142.4 ± 1.4 and 100.8 ± 1.8 erg/mm^2 , respectively). The similar UV sensitivities of B728a and I-1, when suspended in a common culture filtrate, suggest that differences observed between these strains on solid membrane surfaces were due to the extracellular fluorescent pigment rather than other unknown differences that potentially could result from chemical mutagenesis. Bacterial cells were more sensitive to UV exposure on membrane surfaces than in broth medium, presumably because of the shielding effect of the liquid in which the cells were suspended in the latter case. Although the LD_{50} s of both strains varied 10-fold between membrane surfaces and broth medium, in both cases, the LD_{50} s of cells in the presence of pigment was about 50% greater than those of cells in the absence of pigment.

UV sensitivity on leaf surfaces. Curves describing the loss of cell viability with exposure to UV varied greatly between culture and leaf surface environments (Fig. 2). Viable bacterial cells declined logarithmically by a factor of over 1,000-fold with increased UV exposure in culture (Fig. 2A). On leaf surfaces, the number of viable cells decreased logarithmically until about 90% of the original population size was killed. Numbers were then relatively stable with increased UV exposure (Fig. 2B). About 10% of the cells that could be washed off of leaf surfaces were not influenced by doses of UV irradiation ≤ 824 erg/mm^2 .

Survival of parental and Flu- derivative strains on leaf surfaces was compared over UV doses in which numbers of viable bacterial cells decreased logarithmically with increasing UV exposure. The average fraction of cells not killed by UV was subtracted from the number of viable cells measured at each irradiation level to calculate the number of sensitive cells remaining. This number was used in calculations of LD_{50} s from plots exemplified in Figure 2B. The experiments were carried out on healthy and on iron-chlorotic

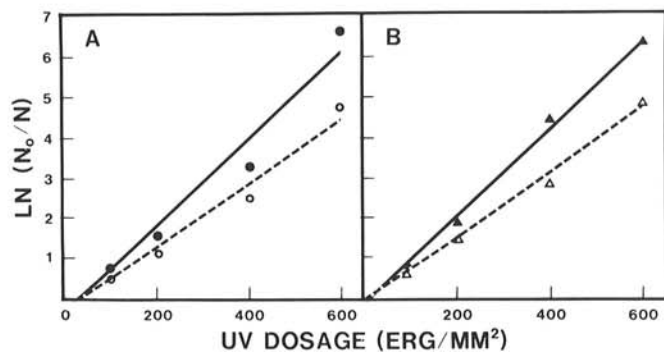


Fig. 1. Effects of the extracellular fluorescent pigment produced by strain B728a in protecting bacterial cells from ultraviolet (UV) irradiation. Cells of **A**, strain B728a (●, ○) or **B**, those of a nonfluorescent derivative strain, I-1 (▲, △), were suspended in culture filtrates of B728a (dashed line) or of I-1 (solid line) and exposed to UV irradiation ($\lambda = 254$ nm).

bean plants, where iron concentrations available for epiphytic bacterial growth were at a minimum. The iron concentrations in healthy and chlorotic bean leaves were 173 ± 9 and 126 ± 12 $\mu\text{g}/\text{g}$, respectively. LD_{50} values calculated from the logarithmic portion of the UV kill curves from four separate experiments indicated that parental and Flu- derivative strains were similar with respect to UV sensitivities on leaf surfaces (Table 1).

Population dynamics on leaf surfaces. The growth rates and stationary population sizes of strain B728a and derivative strain I-1 on leaf surfaces of healthy or chlorotic plants were also similar (Fig. 3). Although the iron nutrition of the plant did have an influence on the growth of both strains on bean leaf surfaces, no significant difference was observed between the strains with respect to growth rate (Table 2). Average population doubling times of both strains calculated from three individual experiments were similar.

Population sizes of four *P. s. pv. syringae* strains and Flu- derivatives of each strain were determined at different times after inoculation of leaf surfaces of field-grown bean plants in four independent experiments. The results of one experiment, presented in Figure 4, are typical of the other three. There was no consistent difference observed between the survival of parental and Flu- derivative strains under field conditions, although individual mutants sometimes differed from parental strains (Fig. 4B,D). In most cases, the Flu- derivatives survived as well as or better than parental strains.

TABLE 1. Sensitivity of epiphytic populations of *Pseudomonas syringae* pv. *syringae* to ultraviolet (UV) irradiation

Experiment	LD_{50} (erg/mm^2) ^a			
	Strain B728a		Strain I-1	
	Healthy plants	Iron-chlorotic plants	Healthy plants	Iron-chlorotic plants
1	97	NT	202	NT
2	182	NT	227	NT
3	198	188	104	76
4	82	NT	98	164
Mean (\bar{x})	140	...	158	...

^a LD_{50} was calculated from the slope of the linear portion of a UV kill curve such as that shown in Figure 2B. No significant differences ($P = 0.05$) were observed among the values of a given experiment or among mean values presented. NT = not tested.

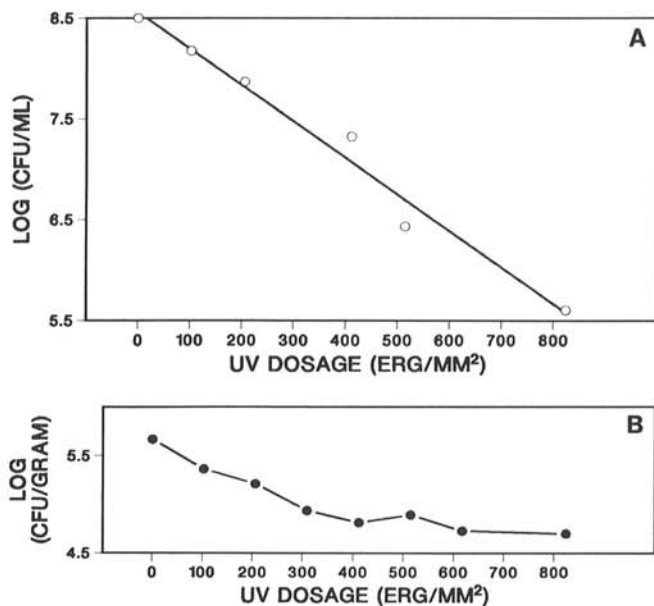


Fig. 2. Effects of ultraviolet (UV) irradiation on viability of *Pseudomonas syringae* pv. *syringae* B728a **A**, in culture and **B**, on bean leaf surfaces.

Brown spot lesion development. Bacterial brown spot lesions developed in the greenhouse 3–5 days after leaflets had been colonized by *P. s. pv. syringae*. Lesion numbers on individual leaflets were quantified in two independent experiments. The mean number of lesions on healthy leaflets (33 and 34 for B728a and I-1, respectively) and on iron-chlorotic leaflets (37 and 32 for B728a and I-1, respectively) were similar statistically among iron and bacterial treatments.

DISCUSSION

Many phenotypes expressed by microorganisms are readily detected and quantified in culture, whereas these same phenotypes may be difficult to detect in natural environments. This is the case with the production by *P. s. pv. syringae* of a fluorescent pigment that functions as a siderophore in culture. No methods have been developed for quantifying *Pseudomonas* siderophores in natural habitats, although such methods for detecting general classes of high-affinity iron-chelating compounds have been described (26). Indirect approaches, such as the construction of mutant strains impaired in the production of fluorescent siderophores, are therefore useful in assessing the production and possible role of such compounds under natural conditions.

The fluorescent siderophore produced by *P. s. pv. syringae*, because of a strong absorption of UV irradiation, conferred significant UV protection to the Flu+ parental strains in culture.

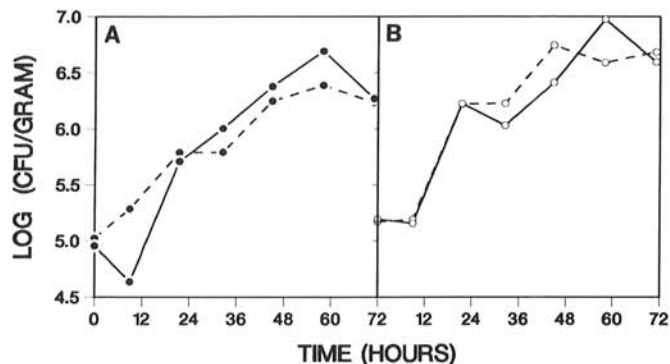


Fig. 3. Mean population sizes of *Pseudomonas syringae* pv. *syringae* A, strain B728a and B, its Flu⁻ derivative, I-1, estimated on healthy plants (solid line) or iron-chlorotic plants (dashed line) maintained in a mist chamber.

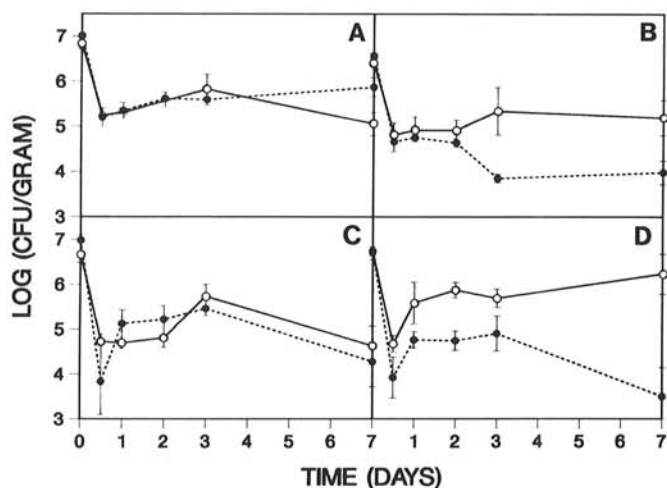


Fig. 4. Mean population sizes of four *Pseudomonas syringae* pv. *syringae* strains (o) and their respective Flu⁻ derivatives (o) on leaf surfaces of field-grown bean plants. Population sizes of strains A, B728a and I-1, B, B728b and O-1, C, J944 and A-2, and D, B733 and P-1 were estimated over a 7-day period. Vertical bars represent standard error ($P = 0.05$) of mean population size.

Nearly all of the *P. s. pv. syringae* cells exposed to UV irradiation on plants in this study had arisen from cell division on leaf surfaces. Bean leaves were irradiated with UV only after a maximum population size had been achieved, at which time only 1% of the bacterial cells present were potentially of the original inoculum (Fig. 3). Thus, cells for which the UV sensitivity was measured represent *P. s. pv. syringae* cells in a natural location and physiological state on leaves. Because bacterial cells of Flu+ and Flu⁻ strains did not differ in susceptibility to UV in the absence of the fluorescent siderophore (Fig. 1), UV susceptibility was used as an indirect score for the presence of fluorescent pigment on leaf surfaces. Because no significant differences between Flu+ and Flu⁻ strains on healthy green bean leaves or on iron-chlorotic plants were observed, no evidence for the production of fluorescent siderophores on plants of either iron nutrition was obtained. *P. s. pv. syringae* strains B728a and its Flu⁻ derivative, strain I-1, showed about the same UV LD₅₀ on leaf surfaces as did the Flu+ strain in broth culture (Table 2). Thus, other UV protectants, such as of plant origin or of bacterial origin (such as extracellular polysaccharide) but induced only in natural habitats, appeared important in the survival of cells on plant surfaces. Shielding was observed in broth culture in contrast to membrane surfaces, but the UV sensitivities of bacterial cells were consistently greater in the absence of the fluorescent pigment. Therefore, a difference in UV sensitivities of Flu⁻ and Flu+ cells on plant surfaces was expected despite the presence of UV protectants. Only about 90% of all *P. s. pv. syringae* cells on leaf surfaces were killed by even relatively high doses of UV irradiation (Fig. 2B), implying that some cells were in locations inaccessible to UV light as has been indicated in other studies (32). Most bacterial cells were killed by UV light, however, indicating that the UV sensitivity of most of the population of Flu+ and Flu⁻ *P. s. pv. syringae* cells was accurately assessed. No difference in the proportions of Flu+ and Flu⁻ *P. s. pv. syringae* cells escaping UV irradiation was observed (Fig. 2B), indicating that no significant difference in the spatial distribution of Flu+ and Flu⁻ cells was likely.

The fluorescent siderophore produced by *P. s. pv. syringae* confers significant protection against lethality caused by UV irradiation under laboratory conditions in which photoreactivation (5) was minimized. Photoreactivation may play a significant role in UV protection under field conditions because of the simultaneous exposure of cells to both UV and visible light. For this reason it was not unexpected that wild-type and Flu⁻ *P. s. pv. syringae* strains established similar epiphytic population sizes after inoculation onto plants grown under field conditions (Fig. 4).

Flu+ and Flu⁻ strains differed with respect to growth on culture medium in which iron was chelated by EDDA, but their growth

TABLE 2. Growth rates of *Pseudomonas syringae* pv. *syringae* strains on bean leaf surfaces and analysis of variance

Experiment	Population doubling time (hr) ^a			
	Strain B728a		Strain I-1	
	Healthy plants	Iron-chlorotic plants	Healthy plants	Iron-chlorotic plants
1	6.70	8.36	7.72	7.50
2	9.10	13.09	9.12	15.05
3	12.50	13.09	11.58	15.84
Mean (\bar{x})	8.85	11.15	9.12	11.58
Source of variation	df	Sum of squares	Observed F^b	
Total	11	113.06	...	
Treatments	3	27.31	3.46*	
Strain	1	2.28	0.85	
Iron	1	23.38	8.90*	
Strain \times iron	1	1.65	0.63	
Experiments	2	69.70	13.33*	
Error	6	15.76	...	

^a Population doubling times were calculated from the slopes of portions of the growth curves representing logarithmic growth.

^b* = F values significant at $P = 0.05$.

rates were similar in SM medium in which iron was available in very low concentrations ($<10^{-7}$ M) and fluorescence of Flu⁻ strains was observed. Flu⁺ and Flu⁻ strains also grew at similar rates on iron-chlorotic bean plants, although these growth rates were less than those on healthy plants (Table 2). Iron concentrations limiting fluorescent pigment production in culture (10^{-4} M or 5.6 $\mu\text{g/ml}$ of Fe) were substantially less than those determined from the healthy or iron chlorotic leaves of this study (173 and 126 $\mu\text{g/g}$ leaf tissue, respectively). Because the concentration of soluble iron available to bacterial growth on leaf surfaces was not determined, it is possible that iron was not limiting to this growth. Changes possibly associated with iron deficiency in bean plants, such as changes in nutrient leakage, may account for the differences in bacterial growth rates observed between iron-chlorotic and healthy plants. Under conditions of iron availability, fluorescent siderophore synthesis would not be expected. Nevertheless, the iron concentrations of the severely chlorotic plants included in this study represent minimum levels that would be encountered under agricultural conditions.

Flu⁺ and Flu⁻ strains represented more than 99% of the total bacteria on treated bean leaves in our experiments (data not shown). Therefore, it is unlikely that contaminating Flu⁺ strains produced a fluorescent siderophore that complemented Flu⁻ strains for iron-limited growth on leaf surfaces. Furthermore, it is unlikely that a microbially produced siderophore, not utilized by *P. syringae*, induced an iron-limited environment on leaf surfaces similar to that induced by EDDA in culture. Siderophores, other than the fluorescent pigment/siderophore, may be produced by *P. s. pv. syringae* and have been described for *P. fluorescens* (34). However, the potential role of other siderophores in iron nutrition of the mutants tested here was minimal because these mutants showed iron-limited growth on agar medium containing EDDA. The possibility exists that nonfluorescent siderophores may be produced by Flu⁻ mutants on leaf surfaces but not in culture. Thus, although our observations do not conclusively demonstrate that siderophores are not produced on healthy or iron-chlorotic bean plants, no evidence for their production was observed.

The evidence for siderophore production by plant-associated prokaryotes in nature remains ambiguous. Siderophores produced by bacteria or algae have been detected in soils by a generalized bioassay (26) or structural characterization (1). Indirect evidence for fluorescent siderophore production on banana leaves has been reported (19). Theoretically, concentrations of iron in banana leaf leachings were sufficiently low to allow siderophore production by a saprophytic *Pseudomonas* sp. Indirect evidence for fluorescent siderophore production in the rhizosphere also exists (10-12,16). Siderophores have been implicated in the pathogenicity of *Erwinia chrysanthemi* (3) but did not appear important in oncogenicity of *Agrobacterium tumefaciens* (14). In the present study, fluorescent pigment production, while influencing iron-limited growth and viability after UV irradiation in culture, did not influence growth, survival, or virulence of *P. s. pv. syringae* on bean leaf surfaces. Although fluorescent pigment production theoretically must play a role in the ecology of *P. s. pv. syringae*, it does not appear to be limiting to the processes on bean leaf surfaces addressed here under the conditions of this study. Fluorescent pigment production is influenced by many nutritional factors, such as carbon source (6,9,18), and physical factors, such as growth temperature (4,18). One or more of these factors may have limited fluorescent pigment production in the phylloplane habitats described in this study. Siderophores may play an important role in specialized niches, such as the rhizosphere, or in specialized processes, such as microbial competition. Hydroxamate siderophores produced by specific blue-green algae reportedly play a role in algal blooms of aquatic ecosystems (2,24). Similarly, the ecological role of microbially produced siderophores may only be exhibited under conditions of intense microbial competition on plant surfaces.

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