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Role of Antibiotic Biosynthesis in the Inhibition of *Pythium ultimum* in the Cotton Spermosphere and Rhizosphere by *Pseudomonas fluorescens*

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Pseudomonas fluorescens **strain Hv37aR2 and its isogenic Afu$^-$ mutants deficient in biosynthesis of an antifungal compound were used to examine the role of an antibiotic in the suppression *in vivo* of *Pythium ultimum* infection on cotton. The results demonstrated that suppression of disease development is dominated by the biosynthesis of an antibiotic(s). An average of 70% of the reduction in root infection and an average of 50% of the increase in emergence caused strain by Hv37aR2 was attributed to the biosynthesis of the antibiotic, oomycin A. Afu$^-$ strains colonized the spermosphere and rhizosphere of cotton as well as their parental strain, indicating that the Afu$^-$ phenotype did not compromise their colonization ability. Seed treatment with strain Hv37aR2 resulted in the formation of significantly fewer *Pythium* propagules in the soil after root infection as compared to Afu$^-$ mutant strain WH103 and the nontreated control. A population level of strain Hv37aR2 of 10^6 cfu per seed at planting was necessary to ensure protection from disease development caused by *Pythium*. Expression *in vivo* of an operon required for antibiotic biosynthesis was determined using a *lacZ* gene (Tn*3*HoHo-1) transcriptional fusion. Gene expression was monitored by measuring β-galactosidase activity in strains growing on cotton seeds. Enzyme activity was readily detectable in the parental strain at 24 h, yet barely detectable in the Afu$^-$ mutant strain. These data demonstrate that the production of an antibiotic(s) *in vitro* was indirectly correlated with biosynthesis of the compound and biological control *in vivo*.**

Additional keywords: antibiotic biosynthesis, biological control, gene expression *in vivo*, gene-reporter system.

Several strains of *Pseudomonas fluorescens* Migula and *P. putida* (Trevisan) Migula have been reported to either suppress infection by soilborne plant pathogens (and subsequent disease development) and suppress the development of other microorganisms that can be deleterious to plant health (Howell and Stipanovic 1980; Kloepper *et al.* 1980a; Suslow 1982; Suslow and Schroth 1982a and 1982b; Weller and Cook 1983; Sneh *et al.* 1984; Weller and Cook 1986; Stutz *et al.* 1986; Leben *et al.* 1987; Loper 1988; Osburn *et al.* 1989). Several mechanisms have been proposed that would explain these phenomena: Biosynthesis of antibiotics (Howell and Stipanovic 1980; Gutterson *et al.* 1986; Brisbane *et al.* 1987; Fravel 1988; Gutterson *et al.* 1988; Thomashow and Weller 1987), hydrolytic enzymes (Sneh *et al.* 1984, Jones *et al.* 1986), production of siderophores (Kloepper *et al.* 1980a, 1980b; Scher and Baker 1982; Marugg *et al.* 1985; Leong 1986; Bakker *et al.* 1987; Simeoni *et al.* 1987; Becker and Cook 1988; Loper 1988), and, lastly, substrate competition (Elad and Baker 1985; Elad and Chet 1987; Paulitz 1990).

To more critically examine the role of antibiotic biosynthesis in disease suppression, we utilized isogenic mutants deficient in antibiotic production of *P. fluorescens* strain Hv37a. The genetics of biosynthesis of one antibiotic (oomycin A) produced by strain Hv37a has been recently reviewed by Gutterson (1990). Strain Hv37a has been shown to suppress the disease development of *Pythium ultimum* Trow on cotton and other crops (T. V. Suslow, unpublished). Preliminary reports on the role of Hv37aR2 and its antibiotic(s) in the suppression of *P. ultimum* suppression have appeared previously (Howie and Suslow 1986, 1987).

Analysis of the role of antibiotics or siderophores in disease suppression in biological control systems has only recently employed the techniques available to a molecular approach (Marugg *et al.* 1985; Loper 1988; Thomashow and Weller 1987). The development of otherwise isogenic strains, deficient only in a single characteristic, is the most efficient means available to resolve the function of an antibiotic in disease control (*in vivo* plant-soil systems). Gutterson *et al.* (1986) characterized several classes of isogenic mutants of strain Hv37a deficient in antibiotic biosynthesis (Afu$^-$). These classes were defined by the genetic complementation analysis of cloned sequences from Hv37a for *in vitro P. ultimum* inhibition. These classes included regulatory and structural mutants comprising in total a minimum of five *afu* genes within four independent operons. Subsequent analysis of Hv37a and its mutants has shown that the only common defect in all five mutants is failure to produce an oomycin A. James and Gutterson (1986) demonstrated that the production of oomycin A in Hv37a is regulated by glucose. Gutterson *et al.* (1988) also determined that the antibiotic biosynthetic operon (*afuE*) is regulated, in part, by its own gene products. An overview of regulation and biosynthesis of oomycin A recently has been described by Gutterson (1990).

The purpose of this study was to determine the role of antibiotic biosynthesis by strain Hv37a in the suppression of disease caused by *P. ultimum* by comparing the disease-

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control ability of parental and isogenic mutants deficient in antibiotic production. We also used a reporter gene (*lacZ*), which is driven by the promoter of the gene segment (*afuE*) involved in antibiotic biosynthesis, to determine gene expression *in vivo* in the background of both the parental strain and an isogenic mutant. Expression of the *afuE* gene is required for the biosynthesis of the antibiotic.

MATERIALS AND METHODS

Soil source, storage, and preparation. The soil used for most studies was a Hesperia fine sandy loam (HFSL) (pH 6.0–6.5) collected either from Porter Farms near Weedpatch, CA, or at the USDA Cotton Research Station, Shafter, CA. An additional study was conducted in a Brentwood Clay Loam (BCL) (pH 6.1) collected from the DNA Plant Technology Corporation field site in Brentwood, CA. At the Brentwood site, the soil was fumigated with methyl bromide in the fall of 1987 and infested with black-eyed peas inoculated with *P. ultimum*. Cotton was planted after approximately four months of incubation and the infested soil was collected at the end of the growing season. Both soils were air-dried and sieved through a 2-mm-mesh screen before being stored at ambient temperature (16–22 C). For some experiments, the HFSL was fumigated with chloropicrin (2 ml/20 kg of soil) to eliminate indigenous plant pathogenic fungi. The HFSL was adjusted to approximately 9.0% moisture or −0.03 MPa before fumigation and then air dried and stored as described above in unsealed containers. Desired soil moisture values were extrapolated from soil moisture retention curves.

Preparation of fungal inoculum. Oospore inoculum of *P. ultimum* was prepared by infesting autoclaved HFSL (at −0.03 MPa) with mycelial plugs of a 1-wk-old culture of *P. ultimum*. Cotton seeds (Acala SJ-2) were then planted into the infested soil and after 2 wk of incubation in the growth chamber (20 C° constant) the soil was air-dried, ground, and sieved through a #20 sieve and stored a 4–8° C. The viable population of the stock was monitored every 2–4 wk by plating 1 ml of a soil suspension (1 g of soil per 100 ml of tap water blended for 1 min) onto a selective medium (MPRM) for *Pythium* spp. (Mircetich and Kraft 1973), which was modified by replacing vancomycin with rifampicin at 50 μg/ml. It had been previously determined that rifampicin at this concentration did not inhibit germination of *P. ultimum* (oospores or sporangia). Appropriate dilutions of the stock inoculum were made with natural or fumigated HSFL to achieve a desired inoculum density, usually 15–25 propagule-forming units (pfu) of *Pythium* per gram in fumigated soil or 100–120 pfu of *Pythium* per gram in natural soil. These inoculum densities resulted in 20–30% emergence of nontreated cotton (standard emergence in pathogen free soil was ≥90%).

Table 1. Bacterial strains used in this study

Strains	Relevant characteristics[a]	Source
Hv37a[b]	*Pseudomonas fluorescens* Afu$^+$, isolated from barley roots	T. V. Suslow
Hv37aR2	Hv37a rif^R, Afu$^+$	T. V. Suslow
WH153	Afu$^-$, *afuB*1912, Hv37aR2::Tn*3503*-12	This study
WH154	Afu$^-$, *afuA*1905, Hv37aR2::Tn*3053*-5	This study
WH164	Afu$^-$, *afuR*2016, Hv37aR2::Tn*3053*-115	This study
WH165	Afu$^-$, *afuE*2008, Hv37aR2::Tn*3503*-8	This study
WH103	Afu$^-$, *afuP*18120, Hv37aR2::Tn*3503*-120	This study
WH108	Afu$^+$, Hv37aR2 (pNG20::Tn*3*HoHo1-142)	This study
WH109	Afu$^-$, WH103 (pNG20::Tn*3*HoHo1-142)	This study

[a] Afu$^+$ exhibits antibiosis *in vitro* against *Pythium ultimum* on potato-dextrose agar. Rif^R exhibits spontaneous resistance to rifampicin. Complementation classes of mutations in *afuA, B, E, R,* and *P* and the β-galactosidase gene fusion (Tn*3*HoHo1-142) were determined by Gutterson *et al.* (1988). Marker exchange experiments were conducted in this study to produce the respective Afu$^-$ phenotype or β-galactosidase fusion in the background of Hv37aR2.

[b] Strain Hv37a exhibits *in vitro* antibiosis on potato-dextrose agar against a broad range of pathogenic fungi. Within the oomycetes, antibiosis against *Pythium ultimum, P. sylvaticum, P. heterothallium, P. aphanidermatum,* and *P. irregulare* has been demonstrated. Antibiosis has also been demonstrated against *Rhizoctonia solani, Thielaviopsis basicola,* and *Fusarium* spp.

Bacterial strains and inoculum preparation. The five strains, carrying mutations representing the different complementation groups *afuA, B, R, E,* and *P* of strain Hv37a (Gutterson *et al.* 1988) were used (Table 1). To facilitate spermosphere and rhizosphere colonization studies we used strain Hv37aR2 (rifampicin-resistant at 80 μg/ml in King's medium B) (King *et al.* 1954) and obtained isogenic mutant strains by site-directed marker exchange (Ditta *et al.* 1980) using the same subclones of cosmids described by Gutterson (1988).

Parental and Afu$^-$ strains were stored in dimethyl sulfoxide (2%, v/v) at −80 C and before each experiment were streaked onto Luria-Bertani agar (Maniatis *et al.* 1982). Several single colonies were grown in Luria-Bertani agar overnight (18 hr) at 28° C, then centrifuged at 3,000 rpm for 15 min, and the pellet was resuspended in 0.01 M phosphate buffer. Bacterial inocula were then prepared to a desired density, usually 10^8 cfu/ml or diluted from 10^8 to 10^4 cfu/ml.

Cotton seeds (Acala SJ-2) were sterilized by soaking them in 10% sodium hypocholorite for 20 min followed by three washes in sterilized distilled H_2O and inoculated by soaking them in the bacterial suspensions for 20 min. Seeds were removed from the suspension and air-dried in a laminar flow hood.

Control seeds were treated with 0.01 M phosphate buffer (pH 7.0) alone or with the fungicide, metalaxyl [*N*-(2,6-dimethylphenyl) *N*-(methoxyacetyl)-DL-alanine methyl ester] (Ciba-Geigy Corporation, Greensboro, NC) at the rate of 50 μl/L. Seeds were soaked and dried as described above.

Assessment of seed and rhizosphere colonization. To enumerate bacterial populations of introduced strains, individual seeds were washed in 9 ml of 0.01 M phosphate buffer then appropriate dilutions were plated onto King's Medium B (King *et al.* 1954) supplemented with 80 μg/ml rifampicin and 50 μg/ml cycloheximide (KMBrc). Populations on seeds were determined at planting for all experiments and for some experiments on days 1 and 2 after planting. Five to 10 seeds were assayed per treatment.

To assess rhizosphere populations of the bacterial strains, a root segment 0–3 cm below the hypocotyl was removed from soil, excised, and vigorously vortexed in 0.01 M

phosphate buffer. Samples (100 μl) of appropriate dilutions were plated onto KMBrc. Eight to 10 roots were assayed per treatment.

Assessment of disease development. *P. ultimum* infection was assessed by determining emergence and the percentage of a root infected. For most experiments emergence was determined at 10–12 days after planting. The percentage of a root infected with *P. ultimum* was determined by plating root segments (0–3 cm below the hypocotyl) onto MPRM that had been first overlaid with 3 ml of 0.2% water agar. After 18 hr roots were scored for infection by counting the number of *P. ultimum* foci emanating from 0.5-cm increments of the segments. This method had previously been validated as comparable to plating individual 0.5-cm sections from a given root (W. J. Howie and T. V. Suslow, unpublished data). The mean value of percentage of a root infected from all the roots (usually five to seven roots/pot) from one pot was considered as a single replication.

Rhizosphere colonization and *Pythium* suppression by parental strains and Afu⁻ mutants. Spermosphere and rhizosphere colonization and suppression of *P. ultimum* were evaluated in three experimental formats: comparison of the five complementation groups; a time-course study from days 1 to 12 using the parental strain HV37aR2 and mutant strain WH103; and a bacterial dose-response study using strain Hv37aR2 and WH103. For comparison of the parental strain Hv37aR2 and respective isogenic mutants, strains were grown and seeds of cotton, Acala SJ-2, were treated with a 10^8 cfu/ml suspension as described above. A natural HFSL or BCL, adjusted to approximately −0.03 and −0.07 MPa, respectively, was used to compare the colonization dynamics and disease suppression of the different complementation groups. The BCL was adjusted to −0.07 MPa for ease of handling. Rhizosphere colonization and disease suppression by strain Hv37aR2 was not significantly different at these two matric potentials between these two soils (W. J. Howie and T. V. Suslow, unpublished data). To compare effective dose thresholds, suspensions of 10^8 and 10^4 cfu/ml were used (the resulting seed populations at planting were 10^6 and 10^2 cfu/seed, respectively). For the time-course study and the dose-response study, seeds were sown in fumigated HFSL adjusted to −0.03 MPa and infested with the appropriate *Pythium* density to give approximately 20–30% emergence as described above. A nontreated control and metalaxyl treatment were also included as appropriate. Plants were grown in 5- or 10-cm^2 pots at 20° C with a 12-hr photoperiod. *Pythium* infection, emergence, and rhizosphere colonization were determined on selected days after planting as described.

The recoverable *P. ultimum* propagules in the bulk and rhizosphere soil were also determined 12 days after planting for soil recovered from the dose-response study. Plants were removed and loosely adhering rhizosphere soil was shaken from the root. All bulk and rhizosphere soil was then thoroughly mixed and allowed to air dry for 10 days. Soil samples from each treatment were taken and the population of *P. ultimum* was determined by the enumeration method described above.

***AfuE* expression *in situ*.** To determine *afuE* expression *in vivo*, an *afuE-lacZ* gene fusion was used as described by Gutterson *et al.* (1988) Gutterson (1990) has demonstrated a positive correlation between *lacZ* activity and antibiotic biosynthesis. The *afuE-lacZ* gene fusion, using Tn*3*HoHo1 (Stachel *et al.* 1985) (pNG20::Tn*3*HoHo1-142) was mated into the background of both the parental strain Hv37aR2 and its mutant WH103 by a triparental mating (Ditta *et al.* 1980). The parental and mutant strains harboring the *afuE-lacZ* gene fusion were denoted as WH108 and WH109, respectively. Strains were grown overnight in Luria-Bertani agar supplemented with tetracycline (20 μg/ml) and seeds of Acala SJ-2 were treated as usual, planted in HFSL at 0.03 MPa, and grown at 20° C. After 24 hr, seeds were removed from the soil and bacterial strains were recovered from the spermosphere of an individual seed in 1 ml of complete Z buffer (Miller 1972). The seeds were then removed from the buffer and the β-galactosidase activity was determined on the retained washing by a standard method (Miller 1972). Bacterial

Table 2. Suppression of *Pythium ultimum* on cotton and rhizosphere colonization by *Pseudomonas fluorescens* strain Hv37aR2 and mutants deficient in antibiotic protection

Seed treatment[a]	Emergence[b] (%)	Infection[b] (%)	Rhizosphere populations (log cfu/3 cm root)[c]
WH153 (*afuB*)	51 c	52 a	4.48 a
WH154 (*afuA*)	47 c	52 a	3.39 b
WH164 (*afuR*)	45 c	52 a	4.49 a
WH165 (*afuE*)	47 c	51 a	3.59 b
WH103 (*afuP*)	51 c	53 a	3.37 b
Hv37aR2	60 b	43 b	3.72 b
Control	36 d	59 a	NA
Metalaxyl	67 a	38 b	NA

[a] Seed were sown in natural Hesperia fine sandy loam that had been infested with 100–120 pfu of *Pythium ultimum* per gram.
[b] Percent emergence and infection was determined on day 12 after planting. Values are the means of three experiments with eight replications or pots (eight seeds planted/pot) per treatment in each experiment and is presented as the arcsine transformation.
[c] Rhizosphere colonization was determined on day 12 after planting. Values are the means of three experiments with eight replications per treatment in each experiment. There was no significant difference between trials so the pooled data are presented for emergence, infection, and colonization.

Table 3. Comparison of the suppression of *Pythium ultimum* on cotton by *Pseudomonas fluorescens* strains Hv37aR2 and WH103 (Afu⁻, *afuP*) in a Hesperia fine sandy loam (HFSL) and a Brentwood clay loam (BCL)

	HFSL[a]		BCL[b]	
Seed treatment	Emergence (%)	Infection (%)	Emergence (%)	Infection (%)
Hv37aR2	59 a	43 b	80 c	20 c
WH103 (*afuP*)	51 b	53 a	59 b	49 b
Control (Ct)	36 c	59 a	32 a	66 a

[a] Data are from Table 2 and are included for ease of comparison.
[b] Seeds were sown in Brentwood clay loam with 80–90 pfu of *Pythium ultimum* per gram of soil. Percent emergence and infection was determined on day 12 after planting. Values are the means of two experiments with seven replications or pots (eight seeds planted/pot) per treatment in each experiment and is presented as the arcsine transformation. There was no significant difference between trials so the pooled data are presented. Mean values followed by a common letter do not differ significantly at $P = 0.05$ as determined by the Duncan's multiple range test.

populations on seeds were also determined at 24 hr by washing a seed in 1 ml of 0.01 M phosphate buffer and then plating the appropriate dilutions onto KMB supplemented with 80 μg of rifampicin and 20 μg of tetracycline per milliliter. Expression of the *afuE* region was normalized as units of β-galactosidase activity/10^8 cfu or as units per seed.

Statistical analysis. All experiments were set up in randomized block design and repeated two or three times. Bacterial populations were analyzed as logarithms (base 10) of cfu/seed or cfu/root segment. Emergence (%) and *Pythium* infection were transformed to their respective arcsine values before analysis because the percentages covered a wide range of values (Steel and Torrie 1980). Statistical analysis was done using analysis of variance, and means were compared using the Duncan's procedure (Statistical Analysis System VMS SAS production release 5.16, SAS Institute, Inc., Cary, NC).

RESULTS

Rhizosphere colonization and *Pythium* suppression parental strain Hv37aR2 and Afu⁻ mutants. All the Afu⁻ mutant classes of Hv37aR2 showed a reduced ability to suppress *P. ultimum* on cotton seedlings with respect to emergence and infection in the HFSL (Table 2). Treatment with the Afu⁻ mutant strains resulted in a significant ($P = 0.05$) decrease by approximately 12% in seedling emergence as compared with the parental strain. Treatment with the mutant also resulted in a significant ($P = 0.05$) increase by approximately 12% in seedling emergence as compared with the nontreated control. A similar pattern was also evident in root infection comparing seeds treated with Hv37aR2, mutants, and untreated control. Seed treatment with the mutants resulted in a significant ($P = 0.05$) 9% increase in infection as compared with Hv37aR2 and nonsignificant 7% decrease in infection as compared to the control. There were no significant differences between Afu⁻ mutants with respect to percent emergence and infection. Seeds treated with strain Hv37aR2 and metalaxyl showed both a significant ($P = 0.05$) increase in emergence and a decrease in infection compared with the nontreated control. Comparison of strains WH103 and Hv37aR2 were conducted in both HFSL and BCL (Table 3). Again, there was a significant ($P = 0.05$) increase (29%) in root infection and significant ($P = 0.05$) decrease by 21% in emergence in seeds treated with the strain carrying the Afu⁻ mutation.

The rhizosphere populations of the Afu⁻ strains WH103, WH154, and WH165 in HFSL and BCL were not significantly different compared with the parental strain. Two Afu⁻ strains, WH153 and WH164, reached population densities that were significantly higher ($P = 0.05$) than Hv37aR2 (Table 2). This difference was reproducible for three separate trials.

To further characterize the biological control potential by strain Hv37aR2 compared with Afu⁻ strain WH103, a study was conducted on the time course of infection. After 1 day approximately 90 and 65% of the nontreated control and WH103-treated seed coats, respectively, were infected with *P. ultimum* (Fig. 1), whereas seed treatment with strain Hv37aR2 resulted in significantly ($P = 0.05$) less infection (16%). By day 4, infection on both the nontreated control and WH103-treated seed coats exceeded 90%, whereas infection frequency after treatment with the parental strain did not exceed 40%. A similar pattern was also evident for the infection of cotyledons (Fig. 1). Root infection, assayed on day 10, was significantly ($P = 0.05$) reduced by the parental strain, and less so by strain WH103 (Fig. 1). The nontreated control plants consistently had the greatest amount of root infection.

Effect of bacterial inoculum density. Spermosphere and rhizosphere colonization and suppression of *P. ultimum* on cotton by strains Hv37aR2 and WH103 were compared at two bacterial inoculum densities on seeds, i.e., approximately 10^2 and 10^6 cfu/seed at planting (Table 4). Spermosphere populations of both strains WH103 and Hv37aR2 increased within 2 days from 10^2 cfu per air-dried seed to approximately 4×10^5 cfu per seed. Spermosphere population densities of strain WH103 were slightly lower (nonsignificant) at 24 hr from 10^2 cfu per seed initial inoculum density; however, by day 10 the

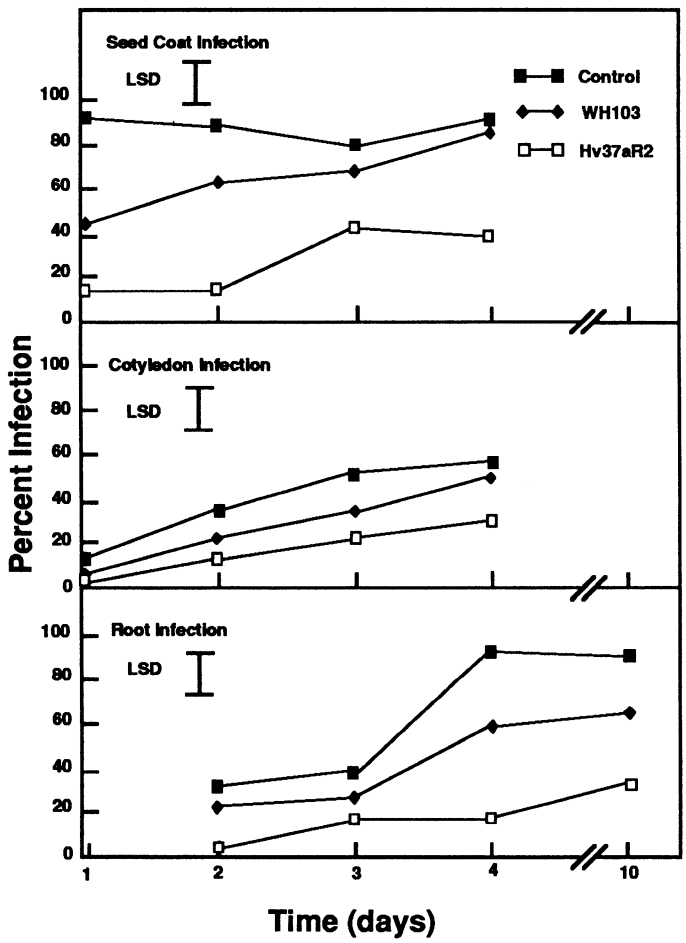

Fig. 1. Effect of seed treatment with *Pseudomonas fluorescens* parental strain Hv37aR2 and Afu⁻ strain WH103 on the suppression *in vivo* of *Pythium ultimum* infection of seed coats, cotyledons, and seedling roots of cotton. Data are presented as the arcsine transformations. Values are the means from two trials with five replications per trial and eight observations per replication. There was no significant difference between trials so the pooled data are presented.

rhizosphere populations of the two strains were the same. Likewise, the spermosphere and rhizosphere populations of strains Hv37aR2 and WH103 inoculated at 10^6 cfu per seed were not significantly different between days 1 and 10.

The populations of the introduced strains (Table 4) in the spermosphere and rhizosphere were significantly ($P = 0.05$) reduced by 10- to 100-fold when seeds had an initial population of 10^2 as compared with 10^6 cfu per seed. Likewise, the low inoculum density on the seed resulted in fewer plants emerging and a higher number of roots infected with *P. ultimum* for both the parental strain and the Afu$^-$ strain (in fact, no significant difference from untreated seed) (Table 4). However, seed treatment with the parental strain at the higher dose resulted in a significant ($P = 0.05$) increase by 12% in emergence and significant decrease by 42% in root infection as compared with seeds treated with strain WH103 (Table 4).

Seed treatment with strain Hv37aR2 resulted in the lowest observed number of recoverable *P. ultimum* propagules in soil, strain WH103 an intermediate level, and the highest densities were observed for the nontreated control (Table 4). This trend was true for initial seed inoculum densities of either 10^2 or 10^6 cfu/seed.

***AfuE* expression *in vivo*.** The induction and expression of β-galactosidase activity in the spermosphere was detected in the Afu$^+$ strain WH108, while little or no expression above background was detected in the Afu$^-$ strain WH109 (Fig. 2). After 24 hr of *in situ* growth on seeds, 75% of the population of strains WH108 and WH107 exhibited retention of plasmid. This was determined by planting onto medium with and without selection (W. J. Howie and T. V. Suslow, unpublished data).

Table 4. Spermosphere and rhizosphere colonization and suppression of *Pythium ultimum* on cotton by *Pseudomonas fluorescens* strains Hv37aR2 and WH103 (*afuP*) applied at difference doses

Seed treatment[a]	Bacterial colonization Log cfu/seed or root[b] Seed Day 1	Seed Day 2	Root Day 10	Emergence[c] (%)	Infection[c] (%)	Secondary *P. ultimum* population[d] (pfu/g soil)
10^2						
HV37aR2	4.56 a	5.81 a	3.10 a	27 a	78 c	1,320 c
WH103	3.85 a	5.44 a	3.11 a	26 a	76 c	2,378 d
10^6						
Hv37aR2	6.49 b	7.23 b	4.71 b	53 c	31 a	375 a
WH103	7.17 b	7.21 b	4.82 b	41 b	73 bc	900 b
Control	NA	NA	NA	28 a	64 b	2,266 d

[a]Seeds were soaked in a bacterial suspension of 10^8 or 10^4 cfu/ml of either strain Hv37aR2 or WH103 (Afu$^-$, *afuP*). After drying, the seed populations were either approximately 10^2 or 10^6 cfu/seed, respectively.
[b]Bacterial populations values are the mean of two experiments with 10 replications per experiment. Mean values followed by a common letter do not differ significantly at $P = 0.05$ as determined by the Duncan's multiple range test.
[c]Percent emergence and percent infection were determined 12 days after planting. Percent emergence is presented as the arcsine transformation. The values are the means of two experiments with 10 replications or pots (eight seeds planted/pot) per experiment.
[d]The population of *P. ultimum* was determined from the rhizosphere and bulk soil 12 days after planting by first removing all plant material from the soil followed by air drying at 30° C for 48 hr. The values are the means of two experiments with 15 replications per experiment. There was no significant difference between trials so the pooled data for emergence, infection, colonization and *P. ultimum* population are presented.

DISCUSSION

We propose that an essential and dominant component in the suppression of *P. ultimum* by strain Hv37aR2 in these studies is the biosynthesis of an antibiotic *in situ* that corresponds, genetically, to oomycin A. The increase in cotton seed emergence by an average of 15% (approximate mean for all experiments) and the decrease in *P. ultimum* infection by an average of 27%, following treatment with strain Hv37aR2 as compared to the Afu$^-$ strains, is attributed to the antibiotic. Alternatively, the contribution of the antibiotic to the biological control of *P. ultimum* can be expressed as an average of 73% of the reduction in root infection and an average of 47% increase in emergence. All of the different Afu$^-$ mutant classes behaved similarly with respect to the significantly reduced suppression of *P. ultimum*. The *afuA, afuB, afuE, afuR,* and *afuP* represent four independent operons (Gutterson *et al.* 1986) in the biosynthesis of the oomycin A. The common defect in all the different mutants is failure to produce oomycin A.

The lack of suppression of root infection by the Afu$^-$ strains was apparently not due to impaired colonization ability because spermosphere and rhizosphere population densities of mutants were not significantly less than those of the parental strain. Surprisingly, two Afu$^-$ strains, WH153 and WH165, had significantly ($P = 0.05$) higher rhizosphere populations than strain Hv37aR2. Even though their rhizosphere populations were higher than for the other Afu$^-$ strains, the level of protection against *P. ultimum* was not significantly different. *In vitro* growth studies in

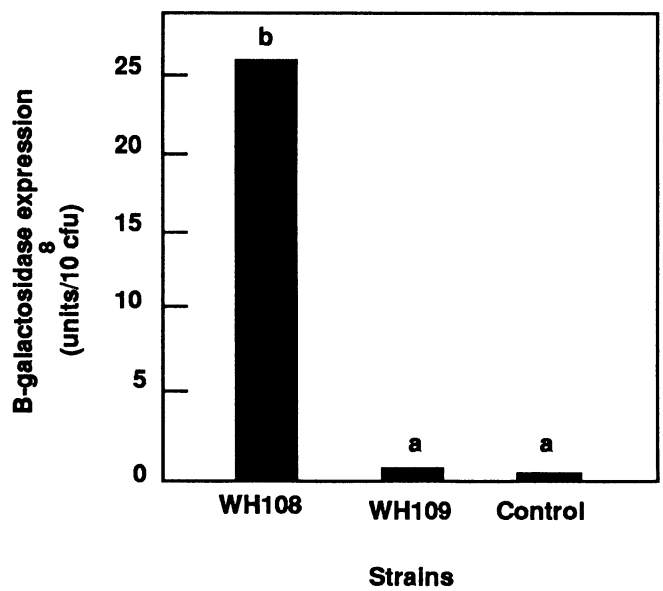

Fig. 2. Levels of β-galactosidase in parental strain WH108 and Afu$^-$ strain WH109 grown on cotton seeds. The values are the means from two trials with 10 replications per trial. Bars labeled with a common letter do not differ significantly at $P = 0.05$ as determined by Duncan's multiple range test. There was no significant difference between trials so the pooled data are presented.

liquid culture showed no differences between mutants and parental strain (W. J. Howie and T. V. Suslow, unpublished data). Other work has also demonstrated that strains carrying mutations that abolish either siderophore or antibiotic biosynthesis, colonized the spermosphere or rhizosphere as effectively as did their parental strains (Bakker *et al.* 1986; Loper 1988; Thomashow and Weller 1987).

Expression of the *afuE* gene *in situ* was detected in the parental strain but not in the mutant strain as measured by β-galactosidase activity. This provides further indirect evidence that expression of gene(s) involved in antibiotic biosynthesis was occurring in the spermosphere environment and indirectly correlates with disease suppression. Expression of the *afuE* region is required for antibiotic biosynthesis (Gutterson 1990).

Residual protection against *Pythium* infection by Afu$^-$ strains was evident from an increase in emergence by an average of 17% and a decrease in infection by an average of 11% compared with nontreated control seeds (Tables 2–4). These values correspond to 27% of the reduction in root infection and 53% of the increase in emergence caused by the parental strain. Both Loper (1988) and Thomashow and Weller (1987) reported that neither siderophore nor antibiotic biosynthesis by *P. fluorescens* strains could account for all of the disease control achieved. It remains to be determined whether other antibiotics or essential nutrient-sequestering (e.g., siderophore) compounds not detected in *in vitro* experimental conditions are produced *in situ* by Afu$^-$ strains. The level of protection observed in the Afu$^-$ strains might be due to competition for nutrients because *Pythium* oospore germination occurs in response to simple sugars such as glucose in seed or root exudates (Lumsden and Ayers 1975; Johnson and Arroyo 1983). The level of protection against disease observed with Afu$^-$ strains was not likely to be caused by a change in the level of expression or regulation of genes involved in biosynthesis of the antibiotic encoded in the *afuE* region since *afuE* expression was not detected in the spermosphere environment (within the detection limits of the β-galactosidase assay used).

We have also demonstrated that for strain Hv37aR2 to be effective against *P. ultimum* a threshold population of bacteria was required. In this study, 10^6 cfu per seed gave effective control of *P. ultimum* on cotton. In this experiment the population of the parental strain and Afu$^-$ strain WH103, applied at 10^2 cfu per seed, reached 10^4 cfu after 24 hr of growth. Once *P. ultimum* had infected cotton seed, with infection beginning approximately 6–8 hr after planting (Howie *et al.* 1988), its inoculum potential would have increased and the bacterial treatment was not capable of controlling subsequent disease development.

In the limited studies presented here, evidence is provided that treatment of cotton seed with strain Hv37aR2 not only reduced disease development during the first 2 wk of seedling growth, but also decreased the secondary inoculum production of *P. ultimum* during the same period. Reducing the *Pythium* inoculum density and perhaps inoculum potential in large-scale applications of bacteria for *Pythium* control may reduce the risk of disease in the following growing season.

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