Take-All Suppressive Properties of Bacterial Mutants Affected in Antibiosis

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ABSTRACT

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Ten Tn*5*-induced mutants of bacterial strains I11 and NRRL B-15135 with altered inhibition (antibiosis) of *Gaeumannomyces graminis* var. *tritici* on agar medium were added to fungal inoculum by vacuum infiltration and tested for their ability to suppress take-all disease. Eight mutants were antibiosis-negative and two mutants gave increased antibiosis. Suppression was evaluated by measuring the weight of plant tops and estimating the level of root infection. In all experiments, all four NRRL B-15135 antibiosis-negative mutants showed lower levels of disease suppression when compared with the parent strain. This was also true with three of the four I11 antibiosis-negative mutants, except in one experiment where, although two of these mutants induced significantly lower plant top weights than those induced by the parent, root infection was not significantly greater. The suppressive properties of the fourth I11 mutant were not significantly different from the parent. The two NRRL B-15135 mutants with increased antibiosis towards the pathogen did not give increased levels of disease suppression.

Additional keywords: biocontrol, biological control.

Take-all of wheat, caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* Walker, is an important disease problem worldwide; there is no economically feasible means of consistent control during continuous cropping of wheat (2). Over a period of 2–3 yr of wheat monoculture, some soils become suppressive to the development of take-all (take-all decline) (13). Gram-negative bacteria have been implicated in this phenomenon (3,5,10, 14,15,19,23). Considerable research efforts have been directed at studying take-all decline, with the objective of developing effective biological control measures (13).

Methods for the screening and identification of biological agents effective in suppression of this and other plant diseases often have included testing for in vitro growth inhibition (antibiosis) of the pathogen as an initial criterion (1,11). Subsequently, antibiosis-positive organisms are tested for their ability to suppress disease. An association between antibiosis and the ability to suppress take-all has been observed, but there are exceptions (not all antibiosis-positive strains are suppressive) (10,15). Thus, the nature of the relationship between the factors responsible for antibiosis and those responsible for disease suppression has been a subject of debate.

The purpose of this study was to determine whether the factors responsible for antibiosis also are involved in disease suppression. We used 10 Tn*5*-induced mutants obtained from two bacterial strains (I11 and NRRL B-15135) that are effective in suppressing take-all (9). These mutants were altered in antibiosis, and each had a single *Eco*R1 Tn*5* insertion fragment. If antibiosis factors are important for disease suppression, then the eight antibiosis-negative mutants should be reduced in suppressiveness, and the two mutants for increased antibiosis might be increased in their suppressive properties.

Take-all suppressive bacteria may prevent infection by either inhibiting pathogen germination in the inoculum propagule (10), or by protecting against infection after colonization of the wheat plant roots (3,18,19). Of the three methods that previously have been used to apply bacteria in tests of suppressiveness (18,19,22,23), infiltration of fungal inoculum with bacterial suspensions is the only method that adds bacteria directly to the infection propagule. Also, strains I11 and NRRL B-15135 introduced in this manner colonized wheat seedling roots to levels of 10^6–10^7 colony-forming units (cfu)/g fresh weight after 3–4 wk (Poplawsky, *unpublished*). Thus, we used the method (inoculum infiltration) that allows bacteria to occupy both of the proposed sites of suppression—the infection propagules and the plant roots.

MATERIALS AND METHODS

Microbial strains and culture media. The two strains of *G. g. tritici* used in this study, Ggt-3 and Ggt-22, have been described previously (9). The parent bacterial strains and the Tn*5*-induced mutants are described in Table 1. Nine antibiosis mutants were prototrophic, and one, 35-4K-13, was auxotrophic (9). Nutrient-broth yeast extract medium (NBY) (17) was used for culture of the bacteria, and potato-dextrose broth (PDB) (250 g/L of potatoes and 20 g/L of dextrose) was used for the culture of *G. g. tritici*. When appropriate, media were supplemented with nalidixic acid and rifampicin, each at 100 μg/ml; or kanamycin at 50 μg/ml.

Preparation of fungal inoculum and biocontrol agents. Strains of *G. g. tritici* were grown in 100 ml of PDB for approximately 4 days. Mycelium then was macerated in a blender, harvested by centrifugation, resuspended in 10 ml of PDB, and added to 250 cm^3 of oat kernels in a 2-L flask. Before the fungus was added, the oat kernels were autoclaved twice in 100 ml of water. The flasks were incubated at room temperature and shaken vigorously two to three times per week. After 3–4 wk, the colonized oat kernels were dried thoroughly under an air stream and then chopped in a blender. Chopped oat kernels were sieved to select pieces 0.36–0.71 mm in size, and this inoculum was stored at 5 C.

Bacterial strains were grown for 20 hr at 28 C in 100 ml of NBY broth supplemented with rifampicin and nalidixic acid (parent strains), or rifampicin, nalidixic acid, and kanamycin (Tn*5* mutants), and then pelleted at 3,000 *g*. Cells were washed once with one-half volumes of sterile water, resuspended in 1–2 ml of sterile water, and mixed with fungal inoculum. This mixture then was placed in a partial vacuum for 12–16 hr, and subsequently dried in a sterile air-stream for 1–2 hr. A 10-mg sample of vacuum-infiltrated fungal inoculum was added to 1 ml of sterile water and vortexed four or five times over the course of 1 hr. Appropriate

dilutions of this mixture were plated on NBY agar medium containing rifampicin and nalidixic acid to determine the number of bacterial colony-forming units.

Growth chamber experiments. These experiments were performed in a manner similar to that of Wilkinson et al (1985) (21). Fungal inoculum was mixed with pasteurized soil (aerated steam at 65 C for 30 min) to a final concentration of 1.4 mg/g. Fifty grams of infested soil was added to a 20-cm cone (Cone-tainer Nursery, Canby, OR) that had been plugged with a rayon puff and half-filled with vermiculite. A randomized complete block experimental design was used. Each experiment contained many treatments, and each treatment contained 14 cones. A pregerminated wheat seed (cultivar Butte) was placed in each cone and covered with a thin layer of vermiculite. Cones were placed in a growth chamber, maintained at 16 C on a 12-hr light/12-hr dark cycle, and watered with Hoaglund's solution every 3–4 days.

After 3–4 wk, plant roots were washed and examined with a Zeiss dissecting microscope at 60× magnification to determine the level of disease. The percentage of roots with lesions induced by *G. g. tritici* and the severity of lower stem infection were estimated. A modification of the disease index of Weller and Cook (19) was used with a rating scale of 0–5, where 0 = no disease and 5 = most severe disease. After excision of the roots, top weights of plants were recorded after drying at 100 C for 24 hr. Differences among treatment means for disease index and plant top weight were tested for significance by Tukey's procedure (comparison of all means) or LSD (comparison of means of mutants to the parent mean).

RESULTS

All mutants derived from strains I11 and NRRL B-15135 initially were selected for changes in antibiosis against fungal strain Ggt-22 (9). It was shown that substitution of Ggt-22 with any of five other strains of *G. g. tritici* had no effect on the antibiosis of either the two parent bacterial strains (9) or any of the antibiosis mutants (Poplawsky, *unpublished*). Thus, the two most virulent fungal strains (Ggt-22 and Ggt-3) were used in these experiments.

It was not known whether moderate differences in bacterial populations in the inoculum preparation would affect the level of disease suppression significantly. Thus, strains I11 and NRRL B-15135 first were grown as described, and then various dilutions were used for vacuum infiltration. The number of bacterial colony-forming units per milligram of fungal inoculum was compared with the level of disease suppression observed. Varying the population density of strain I11 to between 8.4×10^6 and 7.6×10^7 cfu/mg of inoculum had no significant effect on either the average dry weight of the plants or the average disease index (Table 2). Similarly, varying the population density of strain NRRL B-15135 to between 2.5×10^7 and 2.0×10^8 cfu/mg of fungal inoculum had no significant affect on plant weights or disease indexes (Table 2). Replicates of these experiments yielded similar results.

The abilities of strain I11 and four antibiosis-negative mutants of I11 to suppress take-all were compared in two separate experiments (Table 3). In both experiments, the addition of inoculum of *G. g. tritici* treated with sterile water (diseased control) resulted in a high disease index and a large reduction in plant weight compared with the treatment with no fungal inoculum. Treatment of fungal inoculum with parent strain I11 decreased the disease index and increased plant weights in both experiments. None of the treatments with four antibiosis-negative mutants was as effective as the I11 treatment in decreasing disease index, and these differences were significant in most cases. Treatment with three of the four mutants resulted in significantly lower plant weights than treatment with I11, although in all cases these values were greater than those of the sterile water treatment. Plant weights resulting from treatment with mutant I11-3B-24 were not significantly different than those of the I11 treatment in either

TABLE 1. Bacterial strains

Strain	Origin[a]	Antibiosis[b]	Reference or source
Pseudomonas fluorescens			
NRRL B-15135 (35)		+	ARS Patent Collection
35-3C-8	Strain 35; Tn*5* (10.0,9.3)	−	9
35-4C-8	Strain 35; Tn*5* (10.0,9.3)	−	9
35-4K-13	Strain 35; Tn*5* (10.0,6.8)	−	9
35-4M-14	Strain 35; Tn*5* (10.0,4.3)	−	9
35-2M-12	Strain 35; Tn*5* (4.3)	++	9
35-1C-18	Strain 35; Tn*5* (9.2,10.3)	++	9
Gram-negative			
I11		+	6,9
I11-1D-2	Strain I11; Tn*5* (17.3,6.1)	−	9
I11-1K-13	Strain I11; Tn*5* (24.3,3.5)	−	9
I11-3B-24	Strain I11; Tn*5* (10.8,2.8)	−	9
I11-2B-23	Strain I11; Tn*5* (2.2,3.5)	−	9

[a] Antibiosis mutants were produced by Tn*5* mutagenesis (9). The first number in parentheses is the size of the *Eco*R1 Tn*5* insertion fragment, and the second is the size of the *Kpn*1 Tn*5* insertion fragment.
[b] +, wild type level of antibiosis; −, little or no antibiosis; ++, 1.5 to 2× the wild type level of antibiosis.

TABLE 2. The effect of different population densities of bacteria on disease suppression

Strain	Population density (cfu/mg × 10^7)[a]	Average dry weight of plant tops[b]	Average disease index[c]
...	...	119 w	0.0 w
I11	0	45 x	3.5 x
	0.8	69 y	3.4 x
	2.0	70 y	3.4 x
	2.5	64 xy	3.5 x
	7.6	67 xy	3.3 x
...	...	119 p	0.0 p
NRRL B-15135	0	40 q	3.6 q
	2.5	86 r	2.5 r
	4.4	87 r	2.4 r
	7.6	79 r	2.2 r
	20.0	94 r	2.4 r

[a] Number of bacterial colony-forming units found in oat-kernel inoculum colonized by *Gaeumannomyces graminis* var. *tritici* (Ggt-3) immediately after vacuum infiltration with bacterial suspensions.
[b] A separate experiment with 14 replicates per treatment was performed for each strain. For the I11 experiment, Tukey's w ($P = 0.05$) = 24; for the NRRL B-15135 experiment, Tukey's w ($P = 0.05$) = 16. Values within a bacterial strain followed by the same letter were not significantly different.
[c] Scale of 0–5, where 0 = no disease and 5 = most severe disease. For the experiment with I11, Tukey's w ($P = 0.05$) = 0.4; for the experiment with NRRL B-15135, Tukey's w ($P = 0.05$) = 0.4. Values followed by the same letter were not significantly different.

TABLE 3. Effect of strain I11 and antibiosis mutants on the level of take-all disease (*Gaeumannomyces graminis* var. *tritici*) and plant weight

Ggt	Strain	Antibiosis properties[a]	cfu/mg inoculum (× 10^7)[b] Experiment[e] A	B	Average disease index[c] Experiment[e] A	B	Average plant weight (mg)[d] Experiment[e] A	B
−	...	...	...	...	0	0	207	122
+	Sterile water	...	0	0	3.3	4.1	91	34
+	I11	+	1.4	4.0	2.6	3.4	151	61
+	I11-1D-2	−	7.2	5.2	3.2*	3.6	107*	46*
+	I11-1K-13	−	0.3	3.8	3.4*	3.6	113*	52*
+	I11-3B-24	−	1.4	4.8	2.9*	3.6	157	59
+	I11-2B-23	−	...	4.5	...	3.8*	...	52*
	$LSD_{(0.05)}$				0.2	0.3	28	8

[a] +, antibiosis comparable to parent; −, little or no antibiosis.
[b] Bacterial cfu/mg of fungal inoculum.
[c] Scale of 0–5 where 0 = no disease and 5 = most severe disease; * = significantly different than parent value.
[d] * = significantly different than parent value.
[e] In experiment A, *G. g. tritici* strain Ggt-22 was used and the experiment was terminated after 4 wk, whereas in experiment B, *G. g. tritici* strain Ggt-3 was used, and the experiment was terminated after 3 wk.

experiment.

The abilities of strain NRRL B-15135, four antibiosis-negative mutants, and two mutants with increased antibiosis to suppress take-all were compared in two separate experiments (Table 4). In both experiments, treatment of fungal inoculum with strain NRRL B-15135 resulted in a reduced disease index and increased plant weight when compared with treatment with sterile water (diseased control). In all cases, treatment with the antibiosis-negative mutants resulted in significantly higher disease indexes and significantly lower plant weights than the NRRL B-15135 treatment, although in most cases plant weights were higher than the treatment where fungal inoculum was infiltrated only with sterile water. Treatment with either of the two mutants with increased antibiosis also resulted in higher disease indexes than the NRRL B-15135 treatment, although significantly reduced plant weights were observed only in experiment A.

In the above experiments, we estimated disease severity and measured plant weight. Despite the fact that disease indexes are subjective, there was a high negative correlation between average disease severity and average plant weight in these experiments ($r = -0.89$ to -0.98), suggesting that the two measurements reflected the same phenomenon.

DISCUSSION

If antibiosis-negative mutants were less suppressive to take-all than their parent strains, then we expected that the plants treated with these mutants would be reduced in weight and show higher disease indexes than the plants treated with the parent strains. Our plant top weight results show that, when compared with suppressive parent strains I11 and NRRL B-15135, seven of eight antibiosis-negative mutants were significantly reduced in their ability to suppress take-all. The disease index results support this conclusion, except in one I11 experiment (Table 3, experiment B), where the disease indexes of most of the mutant treatments were not significantly ($P = 0.05$) higher than the disease index of the parent. We would expect the measurement of plant top weights on a scale of 0–207 units to more accurately reflect small differences among treatments than the estimation of disease index on a scale of 0–5 units. The differences in suppressiveness between mutants and parent strains were not due to small differences in the number of bacteria applied. In separate experiments with I11 and NRRL B-15135, comparable differences in bacterial numbers had no significant effect on suppression (Table 2). Thus, most of the mutations that abolished antibiosis also reduced take-all suppression. Although this strongly suggests that antibiosis factors are involved in suppression, some of our results were inconsistent with this idea.

One antibiosis-negative mutant, I11-3B-24, gave levels of suppression similar to those observed with its parent, strain I11 (Table 3). Antibiosis by strain I11 depended on a high level of glucose in potato-dextrose agar (PDA) (9). This phenomenon also was observed with *Pseudomonas fluorescens* strain HV37a, which suppresses damping-off of cotton seedlings caused by *Pythium ultimum* (4). Some antibiosis-negative mutants of HV37a no longer showed glucose regulation of antibiotic production and were deficient in the enzyme glucose dehydrogenase. The evidence indicated that other factors besides glucose dehydrogenase were needed for induction of antibiotic biosynthesis. Although antibiosis in I11 was induced by glucose on PDA, it is possible that induction of the antibiosis factor in the root-soil environment is by an alternative mechanism. Thus, mutant I11-3B-24 may be inactivated for glucose-mediated induction on PDA, but unaltered for induction in the root-soil environment. This could explain the suppressive properties of this antibiosis-negative mutant. Because, in contrast to I11-3B-24, the other three I11 antibiosis mutants were reduced in suppression, we would expect them to have insertions in different genes than I11-3B-24. This is likely because the *Eco*R1 and *Kpn*1 fragments carrying the transposon in these three mutants were different than those of I11-3B-24 (Table 1).

Mutants of strain NRRL B-15135 with increased antibiosis did not have increased ability to suppress disease (Table 4). Treatment with these mutants almost always resulted in higher disease ratings than treatment with NRRL B-15135 and did not result in increased plant weights. Although this result was unexpected, similar results were found with avirulent biocontrol strains of *Agrobacterium radiobacter* (12). Mutants that produced increased antibiosis towards the crown gall pathogen on agar medium either were unchanged or reduced in disease suppression when compared with strains showing normal antibiosis.

Although seven antibiosis-negative mutants of strain I11 and NRRL B-15135 were reduced significantly in disease suppression, in most cases some suppression still was observed. Treatments with these strains consistently showed lower levels of root disease and higher plant weights than treatments without bacteria (Tables 3 and 4). Suppressive mechanisms other than those causing antibiosis on PDA might have been responsible for this residual level of suppression. Different antibiotics could be involved since some bacteria produce multiple antibiotics (7), and there also is evidence for iron competition in suppression of take-all (5,8). Other mechanisms, such as site or substrate competition, also might have caused these low levels of suppression (16).

NRRL B-15135 and take-all suppressive *P. fluorescens* strain R1a-80 are the same strain (D. Weller, *personal communication*). In a previous preliminary report, chemical mutants of strain R1a-80, which were selected for a loss of in vitro antibiosis, were less suppressive to take-all than the parent (2,20). It should be noted that our data and conclusions do substantiate this previous report.

We conclude that, with strains I11 and NRRL B-15135, factors responsible for antibiosis are also important for disease suppression as expressed in our growth chamber experiments. But, these strains may have additional mechanisms for suppression not expressed as antibiosis on PDA. Also, our results indicate that, with these two strains and Tn*5*-generated mutants derived from them, there is not a strict relationship between the level of antibiosis on PDA and the level of disease suppression.

TABLE 4. Effect of strain NRRL B-15135 and antibiosis mutants on the level of take-all disease (*Gaeumannomyces graminis* var. *tritici*, Ggt-3) and plant weight

Ggt	Strain	Antibiosis properties[a]	cfu/mg inoculum ($\times 10^7$)[b] Experiment[e] A	B	Average disease index[c] Experiment[e] A	B	Average plant weight (mg)[d] Experiment[e] A	B
−	...	...	...	...	0	0	183	126
+	Sterile water	...	0	0	4.2	3.4	24	51
+	NRRL B-15135	+	7.9	0.9	3.3	0.4	94	127
+	35-3C-8	−	7.9	1.1	3.7*	3.2*	55*	72*
+	35-4C-8	−	3.6	23.0	3.9*	3.3*	45*	50*
+	35-4K-13	−	...	1.4	3.7*	3.0*	64*	77*
+	35-4M-14	−	...	3.0	...	3.0*	...	85*
+	35-2M-12	++	5.2	1.7	3.4	2.1*	58*	116
+	35-1C-18	++	1.0	2.3	3.7*	1.6*	41*	122
	$LSD_{(0.05)}$				0.2	0.3	17	24

[a] +, antibiosis comparable to parent; −, little or no antibiosis; ++, 1.5–2 times the antibiosis of the parent.
[b] Bacterial cfu/mg of fungal inoculum.
[c] Scale of 0–5, with 0 = no disease, and 5 = most severe disease; * = significantly different than parent value.
[d] * = significantly different than parent value.
[e] Experiment A was terminated after 4 wk, whereas experiment B was terminated after 3 wk and 3 days.

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but remain steep along a disease front moving away from the initial