Genetics of Antibiosis in Bacterial Strains Suppressive to Take-All

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ABSTRACT

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We initiated a genetic analysis of the interaction between the fungal pathogen, *Gaeumannomyces graminis* var. *tritici* and bacterial strains involved in suppression of the take-all disease. Tn*5*-induced mutants of three bacterial strains (*Pseudomonas fluorescens* NRRL B-15133, NRRL B-15135, and strain I11) that inhibit the growth of the pathogen on agar media (antibiosis) were obtained. Mutants for auxotrophy (0.67%), increased antibiosis (0.83%), loss of antibiosis (0.38%), and loss of fluorescence on King's Medium B (NRRL B-15135 only, 0.53%) were obtained. When 48 mutants affected in antibiosis were analyzed by Southern blot hybridization, 45 contained insertions in single *Eco*R1 fragments. From these results it was estimated that mutation at six to 10 different loci resulted in a loss of antibiosis. The data suggest that with each strain insertions of at least one half of the prototrophic, antibiosis-negative mutants may be clustered in one or two regions of the bacterial genome. Genomic cosmid clone banks were prepared from two strains (B10 and I21) that did not inhibit the growth of the pathogen on agar media, and transferred to strain NRRL B-15135. One I21 cosmid clone inhibited the expression of antibiosis in this strain, as well as two other strains. Thus, two types of bacterial genes important for the in vitro interaction with the pathogen were identified in this study.

Additional key word: biocontrol.

Take-all disease of wheat (and other cereals), caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici*, is economically important worldwide. Continuous cultivation of wheat on the same land may result in an increase in the incidence and severity of take-all followed by a decline (28). Many different theories have been postulated to explain take-all decline (15,27). Most recent work has concentrated on gram-negative, rhizosphere bacteria (particularly pseudomonads), which provide suppression of take-all (5,19,27,35). Although many suppressive strains inhibit growth of the pathogen on agar media (antibiosis), not all inhibitory strains isolated from soils in which take-all decline has occurred are effective as suppressive agents (27). Thus, the relationship between antibiosis and suppression is unclear. Previous and concurrent work has tested whether specific bacterial products are involved in antibiosis and suppression. Work with the suppressive strain, B10, has implicated *Pseudomonas* iron-chelating siderophores in the antibiosis and suppression of take-all (19). Genetic analyses of siderophore production have been initiated (24,25). In addition, work with *P. fluorescens* strain 2-79 has indicated that a phenazine antibiotic has an active role in suppression of take-all. Five Tn*5*-induced mutants that were negative for antibiotic production were also negative for antibiosis to the pathogen and exhibited reduced suppressiveness to take-all (33).

The objective of this work was to initiate a genetic analysis of the interaction between *G. g.* var. *tritici* and disease suppressive bacterial strains. We postulated that there are specific genes in each microbe that control this interaction, and that there are two types of bacterial genes involved in this interaction. The first type of gene might have an active function to promote suppression. The second type might have an active function to prevent suppression. Because antibiosis has been associated with suppression of the disease (27), we began a mutational analysis of the antibiosis properties of strains effective in take-all control. We wanted to know the phenotypes of mutants that would be obtained, and the approximate number of loci in the bacterium that control antibiosis to *G. g.* var. *tritici*. We reasoned that mutation of genes with an active function for antibiosis would result in a loss of antibiosis, while mutation of genes with an active function to prevent antibiosis would result in increased antibiosis. Transposon mutagenesis was used since single insertion mutations can be identified by DNA blot hybridization. The transposon Tn*5* was selected for these experiments since insertion is comparatively random in *Escherichia coli* and other gram-negative bacteria, including *Pseudomonas* (1,2,24).

We employed a second strategy for the identification of genes with an active function to prevent antibiosis. We reasoned that genes of this type might convert an antibiosis-positive strain to an antibiosis-negative strain. Genomic clone banks, which were constructed from bacterial strains that did not express antibiosis toward *G. g.* var. *tritici*, were transferred to an antibiosis-positive strain, and transconjugants were tested for a change in antibiosis. The inactivation of antibiosis, whether through mutation or the use of cloned sequences, will ultimately be useful in assessing the importance of antibiosis in disease suppression.

MATERIALS AND METHODS

Microbial strains and culture media. Five of the six fungal strains used in this study (Ggt-1, Ggt-4, Ggt-22, CA-1, and AR2) were obtained from Don Mathre (Montana State University) and are from diverse geographical locations in the United States. We isolated the sixth strain, Ggt-3, from diseased wheat in Wisconsin. The bacterial strains used in this study are presented in Table 1. *Pseudomonas fluorescens* strains NRRL B-15133 and NRRL B-15135 were obtained from the Northern Regional Research Laboratory in Peoria, IL, where they were originally deposited by R. J. Cook (Pullman, WA). These strains suppress take-all in the field. Strains I11, I21, B10, and E6 are plant growth-promoting rhizobacteria originally isolated from radish and were obtained from J. Parke (University of Wisconsin-Madison). Strain I11 is effective in suppressing take-all in greenhouse and growth chamber experiments (J. Parke, *personal communication*; A. Poplawsky, *unpublished*).

We used the RP4-derived mobilization system developed by Simon et al (29) to introduce the transposon Tn*5* into three suppressive strains. The suicide vector, pSUP2021, contains the

kanamycin-resistance transposon Tn*5* and the RP4 origin of transfer replication (mob site). Plasmid pSUP2021 is harbored in strain S17-1, which has the RP4 transfer functions integrated into its chromosomal DNA. In the presence of recipient cells, pSUP2021 is mobilized by the chromosomal transfer functions.

Plant-associated bacterial strains were cultured in nutrient broth yeast extract medium (NBY) (34) or King's Medium B (KB) (18), and auxotroph selection was performed on a minimal medium (MAS) (6). *E. coli* cells were cultured in Luria-Bertoni medium (LB) (7). Potato-dextrose agar medium (PDA) was prepared with 250 g/L of potatoes and 20 g/L of dextrose. When appropriate, media were supplemented with ampicillin (Ap) at 200 μg/ml; chloramphenicol (Cm), nalidixic acid (Nal), and rifampicin (Rif), each at 100 μg/ml; kanamycin (Km) at 50 μg/ml; tetracycline (Tc) at 12μg/ml; or mitomycin C (Mit) at 1 μg/ml.

Antibiosis testing. Two different techniques were used to test bacterial strains for antibiosis to the pathogen. In the first, a small plug of fungal inoculum (about 4 mm square) was placed in the center of a petri plate; bacteria from a fresh culture were then streaked at the edge of the plate (usually one or two isolates per plate). In the second, the pathogen was first grown in PDA broth for 3–5 days with moderate shaking. Fungal mycelia were then macerated and adjusted to an $A_{600nm} = 0.6$ by dilution with PDA broth. A 1:50 dilution of the mycelial fragments in molten PDA agar (at 42 C) was used as an overlay for the PDA or KB agar media in petri plates. The medium was allowed to harden for 1–2 hr. Twenty-five bacterial isolates were then transferred to the surface of the plates by means of sterile toothpicks or replica plating techniques. The plates were incubated 4–8 days at 20 C before antibiosis reactions were recorded.

DNA extraction and analysis. *E. coli* plasmid DNA was isolated by standard methods (23). Two different plasmid DNA isolation procedures were used with the other bacterial strains. The first was a rapid alkaline lysis procedure (23), which has been useful in the isolation of *Pseudomonas* plasmids as large as 150 kilobase pairs (kb) (26). The second procedure has been used to isolate *Pseudomonas* plasmids as large as 268 kb (32). Whole cell DNA was isolated from bacterial strains by the method of Keen et al (17). DNA-modifying enzymes were purchased from Promega Biotec Inc. (Madison, WI) and used according to the supplier's instructions. Agarose gel electrophoresis, DNA nick translation, and Southern blot hybridization were performed using standard techniques (7,23,32).

Transposon mutagenesis and mutant selection. Donor (S17-1) and recipient bacterial cells were grown overnight on LB medium at 37 C and NBY medium at 28 C, respectively. Loopfuls of donor and recipient cells were scraped from the plates and mixed in a small volume (0.1 ml) of sterile water. This mixture was then placed on LB medium (4.5% agar) and air dried for approximately 30 min. After a 4- to 5-hr incubation period at room temperature, cells were scraped from the mating plates and resuspended in a small volume of NBY broth. Dilutions were plated on NBY medium containing Km, Rif, and Nal for selection of Tn*5* mutants. After 2 to 3 days, individual colonies were transferred to fresh selection plates (25 per plate), and subsequently transferred to MAS, KB (NRRL B-15135 only), and Ggt-22 fungal overlay plates. Mutants selected from any of these media were purified on KB medium, retested on the above media, and then stored in 15% glycerol NBY broth at −80 C.

Genomic clone bank construction. Clone banks were constructed using a broad host-range cloning system originally developed by Ditta et al (8) and modified by Friedman et al (10). The system consists of a cosmid vector (pLAFR1), which can be mobilized to a wide variety of gram-negative species by the plasmid pRK2013, present in a second strain. Methods for sucrose gradient fractionation of partially digested genomic DNA, DNA ligation, and transduction have been described (23). Dephosphorylation of vector DNA was performed with bacterial alkaline phosphatase (International Biotechnologies, Inc., New Haven, CT) according to the manufacturer's instructions, and extracts for in vitro packaging of cosmids (Promega Biotec, Inc., Madison, WI) were also used according to the manufacturer's instructions.

After transduction of strain HB101, cosmids were mobilized to recipient strains in individual tri-parental matings. Presumptive transconjugants were tested for antibiosis by the fungal overlay method with strain Ggt-22. Isolates that were changed in antibiosis were purified on KB medium and retested for antibiosis. Tc-sensitive isolates were selected on medium lacking tetracycline. The plasmid content of all isolates was confirmed by the rapid alkaline lysis procedure (23).

TABLE 1. Bacterial strains used in this study

Strain	Plasmid	Antibiotic resistance or other traits[a]	Reference or source
Escherichia coli			
HB101	···	Sm, *recA13*	4
HB101	pLAFR1	(Tc)	10
HB101	pRK2013	(Km)	8
S17-1	pSUP2021	Tp,Sm (Km,Ap,Cm)	29
Pseudomonas sp.			
NRRL B-15133	···	Rif, Nal, Ab^+, Sup^+	ARS patent collection
NRRL B-15135	···	Rif, Nal, Ab^+, Sup^+	ARS patent collection
E6	···	Rif, Nal, Ab^-	20,21
B10	···	Rif, Nal, Ab^-, Sup^+	19,20
Gram-negative			
I11	···	Rif, Nal, Ab^+, Sup^+	20,21
I21	···	Rif, Nal, Ab^-	20,21

[a] Symbols in parentheses indicate plasmid-encoded traits. Rif = rifampicin; Nal = nalidixic acid; Km = kanamycin; Tp = trimethoprim; Sm = streptomycin; AP = Ampicillin; Cm = chloramphenicol; Tc = tetracycline; Ab = antibiosis; Sup = suppression to take-all disease of wheat.

RESULTS

Bacterial strain characterization. Strains I11, NRRL B-15135, and NRRL B-15133 were sensitive to Km, Cm, and Ap at the concentrations we used. Spontaneous mutants for resistance to Rif and Nal were selected from each strain for the transposon mutagenesis experiments. With the two methods of plasmid extraction and purification, no plasmids were found in any of these three suppressive strains. A plasmid of approximately 48 kb was found in strain I21.

Strains I11, NRRL B-15135, and NRRL B-15133 inhibited the growth of Ggt-22 on PDA medium by the fungal plug inoculum technique. When these three bacterial strains were tested for antibiosis to the five additional strains of the pathogen, they all caused zones of inhibition of 2–4 mm to each of the fungal strains. The concentration of glucose in the PDA medium affected the amount of antibiosis to Ggt-22 observed with each of the three bacterial strains. Little or no antibiosis was observed at a concentration of 5 g/L, while maximum antibiosis was observed at glucose concentrations of 15 or 20 g/L. In addition, at high glucose concentrations, a yellow diffusible pigment was produced by strains NRRL B-15133 and NRRL B-15135. Strain I11 produced no obvious pigments in any medium. Strains I21 and B10 caused no growth inhibition of any of the fungal strains.

In view of previous work with iron-siderophores of *Pseudomonas* (22), it was desirable to determine if the iron (III) concentration of the medium affected the amount of antibiosis observed. Antibiosis to Ggt-22 was assayed on Kb medium with $FeCl_3$ added at concentrations of 5, 10, 20, 50, and 100 μM. Strain I11 produced no fluorescent pigment and caused no antibiosis on any of these media. Fluorescent pigment production by strains NRRL B-15133 and NRRL B-15135 was iron-regulated. The greatest amount of pigment was observed at an $FeCl_3$ concentration of 5 μM, but there was little or no pigment at 100 μM. These results were in sharp contrast to the fungal inhibition assays. Both NRRL B-15133 and NRRL B-15135 caused zones of antibiosis of about 7–8 mm at all iron concentrations. Strain E6 showed iron-regulation of both fluorescent pigment production and antibiosis. No antibiosis or fluorescent pigment production was observed with E6 at 100 μM $FeCl_3$, but increasing zones of

fungal inhibition and pigment production were observed as the iron concentrations were decreased.

It was important to test each of these three suppressive strains for spontaneous mutation to a change in antibiosis. One thousand random isolates of each strain were compared to the parent isolate for antibiosis to Ggt-22 on PDA medium by the fungal overlay method. None of the NRRL B-15135 or 111 isolates was changed in antibiosis. However, although none of the NRRL B-15133 isolates were reduced in antibiosis, 16 caused zones of inhibition that were one and one half to three times the size of that of the parent isolate.

Tn*5* mutagenesis and mutant selection. Kanamycin-resistant transconjugants were obtained at frequencies of 10^{-6}–10^{-8} per recipient colony-forming unit from each of the three strains. The frequency of spontaneous mutation to Km-resistance in each of these strains was less than 10^{-10}; thus, it can be assumed that more than 99% of the isolates were Km-resistant due to the transfer of pSUP2021. At least 600 Km-resistant isolates from each strain were tested for either Ap or Cm resistance (encoded by pSUP2021 but not Tn*5*). None of the isolates from strains 111 or NRRL B-15133 was resistant to the antibiotics, but approximately 2% of the NRRL B-15135 Km-resistant isolates were also Ap resistant. These isolates were purified and shown to be a mixture of the donor *E. coli* strain and Ap-sensitive transposon mutants.

Tn*5* insertion mutants were tested for the ability to grow on minimal medium, cause antibiosis, and produce fluorescence on Kb medium (NRRL B-15135 mutants only) (Table 2). Auxotrophic mutations occurred at a frequency of 0.43–0.8%, depending on the strain. The frequency of mutation to a loss of antibiosis (antibiosis-negative, Fig. 1A) was from 0.35 to 0.43%, or about 50–100% of the frequency of auxotrophs. With strains NRRL B-15133 and NRRL B-15135 more than one-half of the antibiosis-negative mutants were also auxotrophic. All 111 antibiosis-negative mutants were prototrophic. The frequency of mutation to increased antibiosis (Fig. 1B) was either zero or very low with two of the strains (111 and NRRL B-15135), but very high (2%) with strain NRRL B-15133. This high rate of mutation was expected since the rate of spontaneous mutation of NRRL B-15133 to increased antibiosis was 1.6%. It is likely that the majority of the mutants to increased antibiosis from the Tn*5* experiments were the result of spontaneous mutation. None of the antibiosis mutants of strains NRRL B-15135 and NRRL B-15133 were changed in fluorescence on KB medium, with the exception of antibiosis-negative mutant 35-1R-11. This isolate was fluorescence-negative (Flu^-). Approximately one-third of the Flu^- mutants of NRRL B-15135 were also auxotrophic. When five Flu^- mutants were compared with the parent strain for antibiosis to Ggt-22, no differences were observed.

Characterization of antibiosis mutants by Southern blot hybridization. The Tn*5* suicide vector, pSUP2021, was radiolabeled and used as a probe in Southern hybridization experiments to determine, first, whether each mutant affected in antibiosis had single or multiple insertions; and second, the sizes of the Tn*5* insertion fragments. Plasmid pSUP2021 showed no homology to *Eco*R1 cleaved whole-cell DNA from any of the three parent strains, thus *Eco*R1 digestions of mutant isolate whole cell DNAs were probed with pSUP2021. *Kpn*1 digestions were then used for all the members of each group of mutants that appeared to have a Tn*5* insertion in the same *Eco*R1 fragment. Neither enzyme cleaves within Tn*5* (16). An example of the results of these Southern hybridizations is shown for antibiosis mutants of strain 111 (Fig. 2).

There was a single Tn*5* insertion fragment in each of the 14 antibiosis mutants of NRRL B-15135 (Table 3). In 11 antibiosis-negative mutants, insertions appeared to be in six different-sized *Eco*R1 fragments; the insertions of five of the mutants appeared to be in a single 10.0-kb *Eco*R1 fragment, although further analysis with *Kpn*1 indicated that insertions were in at least two different 10.0-kb *Eco*R1 fragments. Of the four prototrophic, antibiosis-negative mutants, three appeared to have a Tn*5* insertion in the same 10.0-kb *Eco*R1 fragment. Further analysis revealed that two of these three mutants appeared to have an insertion in the same 9.3-kb *Kpn*1 fragment. Each of the three NRRL B-15135 mutants to increased antibiosis appeared to have an insertion in a different-sized *Eco*R1 fragment (Table 3).

Each of the 13 antibiosis-negative mutants of 111 appeared to have a single Tn*5* insertion (Table 4), and these insertions appeared to be in seven different *Eco*R1 fragments. Four mutants appeared to have Tn*5* insertions in the same 17.3-kb *Eco*R1 fragment, and the members of three pairs of mutants each appeared to have insertions in the same-sized *Eco*R1 fragment. When these mutants were further analyzed by *Kpn*1 digestion, it was found that the four mutants with *Eco*R1 insertion fragments of 17.3 kb had *Kpn*1 insertion fragments of two different sizes. The members of two of the three pairs of *Eco*R1 insertion mutants all appeared to have the same 3.5-kb *Kpn*1 insertion fragment.

Of 14 NRRL B-15133, antibiosis-negative mutants, 11 appeared to have single Tn*5* insertions, and three appeared to have double insertions (Table 5). Assuming that only one of the two insertions in the latter three isolates inactivates antibiosis, we conclude that insertions in 11 different *Eco*R1 fragments inactivated antibiosis. If we consider only the six prototrophic antibiosis-negative mutants, insertions were in at least five different *Eco*R1 fragments, and three of these six Tn*5* mutants may have had insertions in the same 16.3-kb *Kpn*1 fragment. When seven NRRL B-15133 mutants to increased antibiosis were analyzed, it was found that six had single insertions and one had a double insertion. All of these insertions were in *Eco*R1 fragments of different sizes (Table 5).

Identification of a DNA segment capable of inhibiting antibiosis. Clone banks were constructed from two antibiosis-negative strains, B10 and I21, using strain HB101 as a host (Table 1). Thirty members of each 1,100-member clone bank were assayed for plasmid content; all isolates contained pLAFR1 with an insert. The average insert size of the I21 clone bank was 25 kb, while that

TABLE 2. Numbers[a] and types of Tn*5* mutants from three take-all suppressive strains of bacteria

Strain	Tn*5* mutants screened (no.)	Auxotrophs[b]	Increased antibiosis	Loss of antibiosis, auxotrophs[c]	Loss of fluorescence, auxotrophs[c]
NRRL B-15133	4,000	32 (0.8%)	80 (2%)	6 (0.15%) 8 (0.2%)	···
NRRL B-15135	3,000	22 (0.73%)	3 (0.1%)	4 (0.13%) 7 (0.23%)	10 (0.33%) 6 (0.2%)
111	3,000	13 (0.3%)	0	13 (0.43%) 0	···

[a] Values in parentheses are frequencies.
[b] These isolates were unchanged in antibiosis.
[c] In each case, the first value is the number of prototrophic mutants, and the second value is the number of auxotrophic mutants.

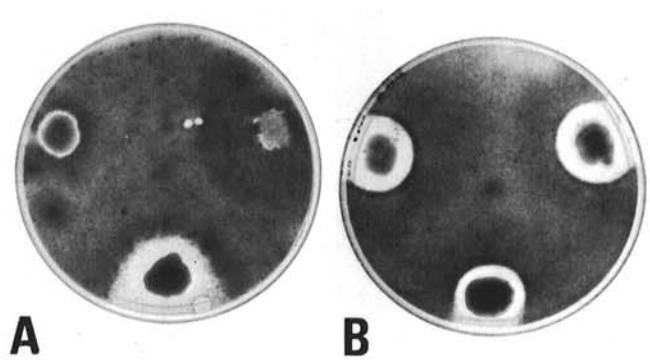

Fig. 1. Growth inhibition of *Gaeumannomyces graminis* var. *tritici* by NRRL B-15135 and Tn*5*-induced mutants. **A,** NRRL B-15135 (bottom), two antibiosis-negative mutants (top), and Ggt-3 (overlay). **B,** NRRL B-15135 (bottom), two mutants with increased antibiosis (top), and Ggt-22 (overlay).

of the B10 clone bank was 22 kb. Each clone bank was replica plated to mitomycin C medium to test for the presence of bacterial genes conferring resistance to the antibiotic. Five members of the B10 clone bank and four of the I21 clone bank were resistant to mitomycin C.

All 1,100 members of each clone bank were individually mated to the antibiosis-positive strain, NRRL B-15135. Transconjugants were compared with their parents for plasmid content. Although all 10 of the B10 clone bank transconjugants assayed contained cosmid inserts identical to those of their *E. coli* parents (as determined by *Eco*R1 digestion analysis), the cosmid inserts in 13 of the 23 I21 clone bank transconjugants assayed were altered (data not shown). Each transconjugant was tested for antibiosis to Ggt-22 by the fungal overlay method. None of the B10 transconjugants was altered in antibiosis, but the transconjugants from one I21 clone (#1089) were reduced in antibiosis (Table 6). I21 clone #1089 contained pLAFR1 with a 23-kb insert composed of three *Eco*R1 fragments; this cosmid was designated p1089. Clone #1089 was mated to suppressive strains NRRL B-15135, NRRL B-15133, and I11, and several transconjugants from each mating were tested for

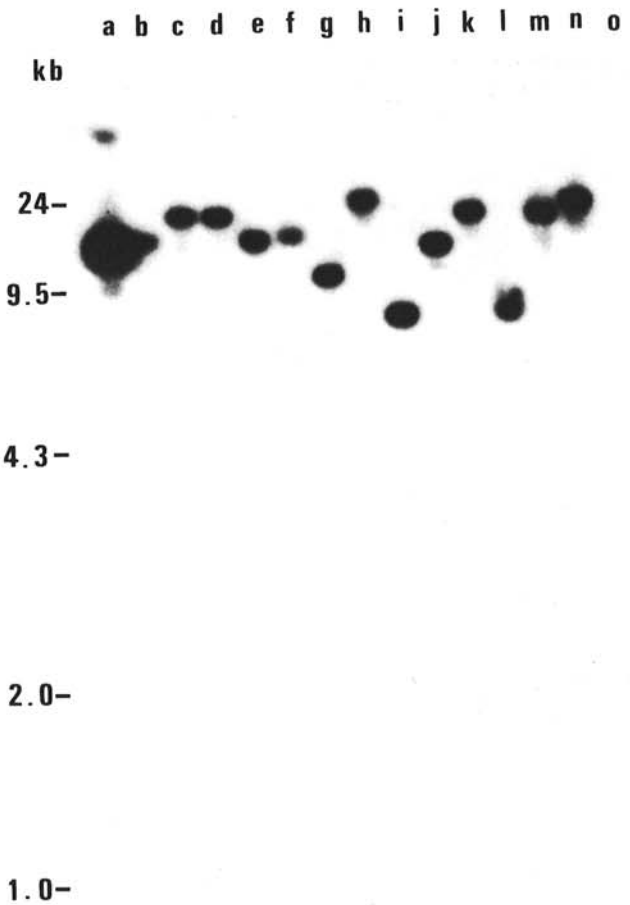

Fig. 2. Southern hybridization analysis of DNAs probed with pSUP2021; Lane a, *Eco*R1-digested pSUP2021 plasmid DNA. Lanes b–n, *Eco*R1-digested whole-cell DNA from 13 different I11 antibiosis-negative mutants; (b) I11-A19, (c) I11-F42, (d) I11-F43, (e) I11-3B-24, (f) I11-4D-24, (g) I11-3E-3, (h) I11-1K-13, (i) I11-2B-23, (j) I11-2C-7, (k) I11-1R-1, (l) I11-2B-24, (m) I11-1D-2, (n) I11-1U-13. Lane o, *Eco*R1-digested whole-cell DNA from strain I11. Note: the upper, light band in lane a is due to incomplete digestion of pSUP2021.

TABLE 3. Phenotypes and summary of Southern hybridization analyses of NRRL B-15135 Tn*5* mutants affected in antibiosis

Isolate	Size of insert fragment[a]		Antibiosis[b]	Growth on minimal medium
	*Eco*R1	*Kpn*1		
NRRL B-15135	...	...	+	+
35-3C-8	10.0	9.3	−	+
35-4C-8	10.0	9.3	−	+
35-4K-13	10.0	6.8	−	−
35-4M-14	10.0	4.3	−	+
35-5B-6	10.0	23.3	−	−
35-3G-21	3.5	2.4	−	−
35-4J-7	3.5	4.8	−	−
35-20-3	12.3	...	−	+
35-1R-11	6.3	...	−	−
35-4J-22	1.2	...	−	−
35-5A-21	7.3	1.8	−	−
35-1C-4	7.3	17.3	++	+
35-1C-18	9.2	10.3	++	+
35-2M-12	4.3	...	++	+

[a] Size of fragment in which Tn*5* inserted (assuming Tn*5* is 5.7 kb).
[b] +, antibiosis comparable to wild type; −, little or no antibiosis; ++, 1.5–2 times the wild type antibiosis.

TABLE 4. Phenotypes and summary of Southern hybridization analyses of I11 Tn*5* mutants affected in antibiosis

Isolate	Size of insert fragment[a]		Antibiosis[b]
	*Eco*R1	*Kpn*1	
I11	...	...	+
I11-1K-13	24.3	3.5	−
I11-1U-13	24.3	3.5	−
I11-2B-23	2.2	3.5	−
I11-2B-24	2.2	3.5	−
I11-F42	17.3	4.3	−
I11-F43	17.3	4.3	−
I11-1R-1	17.3	6.1	−
I11-1D-2	17.3	6.1	−
I11-A19	10.8	2.8	−
I11-3B-24	10.8	2.8	−
I11-4D-24	11.3	...	−
I11-2C-7	9.8	...	−
I11-3E-3	5.3	...	−

[a] Size of fragment in which Tn*5* inserted (assuming Tn*5* is 5.7 kb).
[b] +, antibiosis comparable to wild type; −, little or no antibiosis.

TABLE 5. Phenotypes and summary of Southern hybridization analyses of NRRL B-15133 Tn*5* mutants affected in antibiosis

Isolate	Size of insert fragment[a]		Antibiosis[b]	Growth on minimal medium
	*Eco*R1	*Kpn*1		
NRRL B-15133	...	...	+	+
33PL2	2.2	16.3, 4.1	−	+
33PL8	2.2	16.3	−	+
33PL1	9.0	16.3	−	+
33PL10	9.0	16.3	−	−
33PL5	6.8	41.0	−	−
33PL11	6.8	4.1	−	+
33PL3	5.3, 3.2	...	−	+
33PL4	7.9, 0.35	...	−	−
33PL6	4.3	...	−	+
33PL7	12.1	...	−	−
33PL9	22.1	...	−	−
33PL12	10.3	...	−	−
33PL13	1.2	...	−	−
33PL14	13.2	...	−	−
33PI201	5.7	...	++	+
33PI202	13.5	...	++	+
33PI203	7.6	...	++	+
33PI204	6.0, 0.4	...	++	+
33PI205	3.7	...	++	+
33PI206	6.8	...	++	+
33PI207	26.2	...	++	+

[a] Size of fragment in which Tn*5* inserted (assuming Tn*5* is 5.7 kb). Some isolates harbor two insertions.
[b] +, antibiosis comparable to wild type; −, little or no antibiosis; ++, 1.5–3 times the wild type antibiosis.

antibiosis. All transconjugants contained unaltered p1089 cosmids and were reduced in antibiosis. Spontaneous tetracycline-sensitive derivates of transconjugants from each of these matings contained no plasmid DNA (data not shown) and were restored in antibiosis (Table 6).

Cosmid p1089 transconjugants were tested for other changes, in addition to antibiosis. Transconjugants of strains NRRL B-15133 and NRRL B-15135 were unaltered in fluorescent pigment production on KB medium. In addition, p1089 transconjugants of all three strains were tested for antibiosis to each of the six different *G. g.* var. *tritici* strains. Cosmid p1089 inhibited the expression of antibiosis of all three bacterial strains to all six fungal strains. Thus, the specific fungal strain used to test antibiosis had no effect on the inhibitory activity of p1089.

DISCUSSION

We have presented evidence for two types of bacterial genes involved in the in vitro interaction between *G. g.* var. *tritici* and soil-inhabiting bacteria. Genes with an active function for antibiosis were identified by Tn*5* mutagenesis, while evidence for genes with an active function to repress antibiosis was generated from genomic clone bank experiments. There are five lines of evidence that argue that the antibiosis phenomenon we have analyzed is separate from the effects of fluorescent siderophores or other iron (III) transport agents. 1) We selected for antibiosis mutants on a medium that is not a low iron medium, thus we would not expect to obtain mutants in a system that is iron-antagonized. 2) Two of the three suppressive strains showed iron-regulated fluorescent pigment production, but none of them showed iron-regulated antibiosis to Ggt. 3) Twenty-four of the 25 antibiosis-negative mutants in the two fluorescent strains were not visibly altered in fluorescent pigment production. 4) Five Flu^- mutants of NRRL B-15135 showed the same antibiosis properties on PDA as the parent strain. 5) Cosmid p1089 inhibited the expression of antibiosis but not fluorescent pigment production in strains NRRL B-15133 and NRRL B-15135.

The RP4-derived mobilization system for Tn*5* mutagenesis was very effective with these three suppressive strains. Purified kanamycin-resistant transconjugants were sensitive to plasmid-encoded antibiotic resistance in all cases. This indicates that kanamycin resistance was due to transposition of Tn*5* and not the autonomous replication or chromosomal integration of the suicide vector, pSUP2021. While no spontaneous antibiosis-negative mutants were observed ($<0.1\%$), the rate of mutation to a loss of antibiosis with Tn*5* mutagenesis was 0.38%. The rate of spontaneous mutation to increased antibiosis was approximately the same as that observed with Tn*5* mutagenesis. Thus, although the antibiosis-negative mutants appeared to be the result of Tn*5* insertion, most of the mutants to increased antibiosis were probably unrelated to Tn*5* insertion.

Four of 21 NRRL B-15133 mutants appeared to have multiple insertions (Table 5). Three mutants had insertions in two different *Eco*R1 fragments while a fourth mutant, 33PL2, apparently had two insertions that were in the same *Eco*R1 fragment but different *Kpn*1 fragments. Double insertion mutants may result from either a secondary transposition of Tn*5* or a transposition of one of the IS50 ends of Tn*5*, which are capable of transposing independently (3). Thus, since 5.7 kb (the size of Tn*5*) was used to calculate the size of insertion fragments, some of these values for the double insertion mutants may not be correct. Transposition of the IS50 ends of Tn*5* has been reported in previous experiments with *Pseudomonas* (1,31). Although secondary transposition can cause complications in the isolation of genes identified by these methods, the low frequency of this event in our work should not cause major problems.

A number of different types of bacterial mutations could result in a loss of antibiosis. If the antibiosis factor is a diffusible molecule, then mutations that affect the bacterial cell membrane or wall might interfere with the extrusion of this factor and other factors (such as fluorescent pigment) into the medium. Mutant 35-1R-11 (Table 3) was an antibiosis-negative mutant that was also Flu^-, auxotrophic, and appeared to have a single Tn*5* insertion. It is possible that this insertion caused an alteration in the bacterial cell membrane or wall, and the resulting pleiotropic phenotype. Additionally, if the antibiosis factor is a secondary metabolite, it is possible that some auxotrophic mutations would result in insufficient quantities of a primary metabolite needed for its synthesis. With strains NRRL B-15135 and NRRL B-15133, 15 of the 25 antibiosis-negative mutants were also auxotrophic, and all but one of these mutants appeared to have a single insertion mutation. A third type of mutation might be in genes that have a role in the synthesis of an antibiosis factor, but not in primary metabolism. The prototrophic, antibiosis-negative mutations might be in genes of this type, which will be referred to as genes for specific antibiosis.

The analysis of antibiosis-negative mutants allowed us to estimate the number of different genes in each strain which, when inactiviated, result in a loss of antibiosis. The following assumptions were made; first, at least one gene is associated with each different-sized restriction fragment with an insertion; second, although two mutants may have insertions in different restriction fragments of one enzyme, if the insertions appear to be in the same fragment with a second enzyme, then they may be in the same gene. With these assumptions the following estimates can be made: 1) Strain 111 has at least six different genes for specific antibiosis. 2) Mutations in at least seven different genes resulted in a loss of antibiosis from strain NRRL B-15135, and it has at least two genes specific for antibiosis. 3) Mutations in at least 10 different genes resulted in a loss of antibiosis in strain NRRL B-15133, and this strain contains at least four genes for specific antibiosis. Thus, the inactivation of at least six to 10 different genes resulted in a loss of antibiosis, and specific antibiosis is encoded by a minimum of two to six different genes. With each strain, at least one half of the antibiosis-negative, prototrophic mutants had insertions in one or two fragments of the same size (17.3- and 3.5-kb fragments for 111, a 10.0-kb *Eco*R1 fragment for NRRL B-15135, and a 16.3-kb *Kpn*1 fragment for NRRL B-15133, Tables 3–5). These may be genomic regions that encode multiple genes specific for antibiosis; thus, our estimates are minimum values. Another genetic study has resulted in an estimate of at least five genes in three distinct chromosomal locations for the expression of antibiosis by *P. fluorescens* strain HV37a to *Pythium ultimum* (14). Thus, our estimates are in agreement with the results of this work, which indicates that multiple genes have an active function for the expression of antibiosis.

TABLE 6. Antibiosis of parent strains, p1089 transconjugants, and their plasmidless derivatives

Strain	Antibiotic resistance[a]	Plasmid	Antibiosis (mm)[b]
111	Rif,Nal	···	2.8
111-A1	Rif,Nal,Tc	p1089	0
111-A1-S[c]	Rif,Nal	···	2.6
111-C1	Rif,Nal,Tc	p1089	0
111-C1-S[c]	Rif,Nal	···	2.8
NRRL B-15133	Rif,Nal	···	4.2
33-B2	Rif,Nal,Tc	p1089	0
33-B2-S2[c]	Rif,Nal	···	4.0
33-C1	Rif,Nal,Tc	p1089	0.9
33-C1-S2[c]	Rif,Nal	···	3.9
NRRL B-15135	Rif,Nal	···	4.3
35-3	Rif,Nal,Tc	p1089	0
35-3-S[c]	Rif,Nal	···	4.2
35-4	Rif,Nal,Tc	p1089	0
35-4-S[c]	Rif,Nal	···	3.4

[a] Rif = rifampicin; Nal = nalidixic acid, Tc = tetracycline.

[b] Zones of bacterial inhibition of Ggt-22 on potato-dextrose agar medium were measured with a Wild dissecting microscope with a micrometer eyepiece at a total magnification of 60×. Values are averages of two replicates.

[c] These are plasmidless derivatives of the p1089 transconjugants.

Bacterial genes with an active function to prevent antibiosis might have a regulatory function in the biosynthesis of an antibiosis factor. Alternatively, such genes could specify an interaction with *G. g.* var. *tritici* for a lack of antibiosis—just as avirulence genes specify the interaction of a parasite with a host plant for a lack of virulence (9,30). One would expect insertional inactivation of these genes to result in increased antibiosis. Mutants to increased antibiosis were observed with two of the three suppressive strains, although it is likely that the majority of these mutations were spontaneous in nature. The true nature of these mutations cannot be elucidated until the wild-type copies of the insertion fragments are cloned and used in complementation studies.

We reasoned that the lack of antibiosis in strains B10 and I21 might be due to genes with an active function to prevent antibiosis, and that this activity might be expressed in the antibiosis-positive strain, NRRL B-15135. Assuming an average genome size of 3.5×10^9 daltons for *P. fluorescens* (13), it was estimated that approximately 1,100 members of the B10 clone bank, and 1,000 members of the I21 clone bank would need to be analyzed. These numbers would allow us to be relatively sure ($p = 0.99$) that any given sequence would be represented in our clone banks (23). Clone banks are commonly screened for the presence of a random unselected gene as a measure of completeness. Mitomycin C sensitivity is associated with recombination-deficient strains such as *E. coli* HB101 (11,12), and selection for mitomycin-resistance has been used to isolate a *Pseudomonas syringae* pv. *syringae recA* gene (36). Our clone banks were constructed from two mitomycin-resistant strains (B10 and I21) in strain HB101. Thus, the presence of four or five mitomycin-resistant clones was an indication of the completeness of each of these clone banks. The mitomycin-resistant clones from each strain contained some common *Eco*R1 fragments (data not shown) which probably encoded functional *recA* genes. It was also important to determine whether cosmid inserts were altered after conjugal transfer to strain NRRL B-15135. Although none of the B10 cosmid inserts appeared altered, over one half of the I21 cosmid inserts were altered after transfer. Thus, it is less likely ($p < 0.99$) that any given sequence would be present in the I21 clone bank after transfer to NRRL B-15135.

I21 cone #1089 inhibited the expression of antibiosis in strain NRRL B-15135. None of the other I21 clones, or pLAFR1 itself, affected the expression of antibiosis in this strain. In addition, when p1089 was cured from this strain the level of antibiosis was similar to that of the parent strain (Table 6). This is strong evidence that the I21 insert of p1089 was responsible for inhibiting the expression of antibiosis. This effect was also observed with two other antibiosis-positive, suppressive strains, NRRL B-15133 and I11 (Table 6). The p1089 activity may be due to a specific effect, either directly on the expression of antibiosis in these strains, or indirectly through their interaction with strains of *G. g.* var. *tritici*. Alternatively, p1089 may have a more general effect on the metabolism of these strains, which results in a loss of antibiosis. Because p1089 inhibited the expression of antibiosis to all six strains of *G. g.* var. *tritici*, we have no evidence for a specific interaction between the p1089 activity and strains of the pathogen. Also, since p1089 appeared to have no effect on the production of the fluorescent, diffusible pigments of strains NRRL B-15135 and NRRL B-15133, at least this aspect of the general metabolism of these strains appeared unchanged. It will be interesting to determine whether the p1089 activity is responsible for the lack of antibiosis of strain I21. If this were so, then the replacement of this gene(s) in I21 with an inactivated copy should result in the expression of antibiosis.

It is anticipated that the genes identified in this work will be important in the future analysis of take-all suppression. Seven of eight strain I11 and NRRL B-15135 antibiosis-negative mutants were significantly reduced in suppressiveness when tested in growth chamber experiments (Poplawsky, *unpublished*). The future isolation of genes for the expression of antibiosis, by clone bank complementation of these antibiosis mutants, may allow the enhancement, or transfer to different strains, of this biologically important trait.

Note added in proof: We recently learned (David M. Weller, personal communication) that strains NRRL B-15135 and RIa-80 are the same (see Weller et al, Phytopathology 75:1301).

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