Lack of Correlation of In Vitro Antibiosis with Antagonism of Ice Nucleation Active Bacteria on Leaf Surfaces by Non-Ice Nucleation Active Bacteria

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ABSTRACT

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Only 58% of 88 bacterial strains antagonistic to ice nucleation active strains of *Pseudomonas syringae* and *Erwinia herbicola* on leaf surfaces produced compounds inhibitory to *P. syringae* on any of several culture media tested. Eighty-two percent of those antagonistic to *P. syringae* in vitro produced iron-regulated antibiotics, 6% produced trypsin-sensitive antibiotics, and 39% produced trypsin or iron-insensitive antibiotics that were inhibitory to *P. syringae*. Thirty-one percent of the bacterial strains antagonistic to *P. syringae* on rich culture media were not inhibitory when grown on a defined medium similar in composition to leaf leachates. Frost injury to corn plants treated with mutant strains deficient in production of one or more inhibitory compounds and derived from 24 of 25 parental antibiotic-producing strains did not differ significantly from corn treated with parental strains. Leaf surface populations of antibiotic-deficient mutants generally did not differ significantly from their parental strain, whether applied 2 days before or immediately after challenge inoculations of *P. syringae*. *P. syringae* populations and the numbers of bacterial ice nuclei were significantly lower on plants treated with antibiotic-deficient mutants or their parental strains either two days before or immediately after challenge inoculations with *P. syringae*. Antibiotic production by epiphytic bacteria on several laboratory media is not correlated with biological control of ice nucleation active bacteria on plants.

Additional key words: siderophore, *Zea mays*, phylloplane.

Many pathovars (13) of *Pseudomonas syringae* van Hall and strains of *Erwinia herbicola* (Löhnis) Dye and *Pseudomonas fluorescens* Migula exhibit ice nucleation activity at temperatures above −5 C (1,22–24,37–41,46,47,62). Ice nucleation active (INA) strains of *P. syringae* and *E. herbicola* incite frost damage to many plants (1,35,37,39–41). Most frost-sensitive plants have the capacity to supercool (7) and do not have intrinsic ice nuclei active above −5 C (41). Leaf surface populations of these bacterial species limit supercooling by initiating damaging ice formation at temperatures of −1 to −5 C (37,39–41). Frost injury to plants at temperatures between −1 to −5 C is related directly to the logarithm of the number of INA bacteria, or the number of bacterial ice nuclei on plant parts at the time of freezing (41).

Frost injury to plants is reduced by chemical or biological treatments which decrease either the numbers of INA bacteria or the number of bacterial ice nuclei on plants (35–39,41–44). The application of antagonistic non-INA bacteria will protect plants from subsequent frost injury incited at temperatures above −5 C (35,36,38,42–44). Properties of only a few non-INA bacterial antagonists have been studied. Some but not all non-INA bacteria antagonistic to INA bacteria on plants are also inhibitory to these bacteria in vitro (35,36).

Fungal and bacterial plant pathogens are inhibited in vitro as well as in vivo by some biological control agents (2–4,6,8–11,14–21, 25–27,31–33,45,49–55,57,58,61). However, not all bacteria or fungi applied to plant foliage as biological control agents of plant disease exhibit in vitro antibiosis towards the foliar pathogen (6,9–11,19,21,26,45,50,53). Although antibiotic, siderophore, or bacteriocin production has been reported to be important in interactions of microorganisms in the rhizosphere (29,30,33,60) and is widely used to screen potential biological control agents of both foliar and root diseases, little is known of its importance in the interactions of microbes on leaves. Although it is known that exogenously applied bacteriocins can achieve control of leaf surface bacteria, it is not known if they are responsible for control by producing organisms (2–4,60). This study was designed to investigate the role of antibiotic production by bacteria effective in biological control of epiphytic INA bacteria.

MATERIALS AND METHODS

Media and cultural conditions. All bacterial strains were stored on slants of nutrient agar containing 2.5% glycerol (NGA) at 5 C and were routinely cultured on King's Medium B (KB) (28) containing cycloheximide (100 μg/ml) (KBC) at 21 C unless otherwise indicated. KBC supplemented with rifampicin (100 μg/ml) (KBCR), chloramphenicol (20 μg/ml) (KBCC), streptomycin (100 μg/ml) (KBCS), oxytetracycline (50 μg/ml) (KBCO), or nalidixic acid (100 μg/ml) (KBCN) was used to select one or more components of mixed bacterial populations. A mineral salts medium (MM) containing K_2HPO_4 (3.5 g), KH_2PO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $(NH_4)_2SO_4$ (1.0 g), glycerol (5.0 g), and purified agar (15.0 g) per liter distilled water was used to test bacterial strains for antibiotic production. The production of inhibitory compounds also was evaluated on a synthetic leaf diffusate medium (LDM) similar in composition to that reported by Deverall (12). The composition of full-strength LDM was as follows: sucrose (3.0 g); fructose (1.0 g); glucose (1.0 g); galacturonic acid (0.1 g); glucuronic acid (0.02 g); asparagine (0.03 g); glutamine (0.05 g); aspartic acid (0.11 g); glutamic acid (0.08 g); proline (0.02 g); leucine (0.01 g); KH_2PO_4 (0.1 g); $MgSO_4$ (0.05 g); purified agar (Difco) (15.0 g); distilled water (1 L). LDM medium was adjusted with NaOH to pH 6.8 before autoclaving.

Bacterial strains. The biochemical characteristics and ice nucleation activity of *P. syringae* strain 31R1 and *E. herbicola* strain 26SR6-2 isolated from the surface of healthy corn leaves have been reported previously (38,39). Strain 31R1 is resistant to 100 μg/ml rifampicin and was not pathogenic on any of 11 indicator plants tested (38). Therefore no pathovar designation

(13) for this *P. syringae* strain will be given. The sources, epiphytic competence, and antagonistic characteristics exhibited by several bacterial strains toward INA bacteria have been reported previously (35,36,42,44). Additional bacterial strains were isolated from leaf surfaces of bean (*Phaseolus vulgaris* L.), potato (*Solanum tuberosum* L.), avocado (*Persea americana* Miller), tomato (*Lycopersicon esculentum* Miller), pear (*Pyrus communis* L.), navel orange (*Citrus sinensis* L.), or almond (*Prunus amygdalus* Batsch). Bacterial colonies representative of the most numerous inhabitants on these plants were obtained from dilution plating of leaf or flower washings as described previously (40) onto KBC. The characteristics of *Escherichia coli* strains AN193 and AN194 used to detect siderophore production in this study have been reported previously (34). Spontaneous mutants of antagonistic bacteria resistant to rifampicin (100 μg/ml) and either streptomycin (100 μg/ml), nalidixic acid (100 μg/ml), or oxytetracycline (50 μg/ml) were obtained by spreading approximately 10^9 cells per plate of KBCR, and subsequently plating a similar number of cells on KBCS, KBCN, or KBCO and obtaining single colonies. Antibiotic-resistant mutants of each of these 88 bacterial isolates were also evaluated for their ability to colonize field-grown potato, tomato, pear, almond, or navel orange plants and to reduce frost injury by reducing populations of INA bacteria on these plants under field conditions as in other studies (35,36,42–44) and in greenhouse tests described below. Strain 31R13C is a spontaneous mutant of *P. syringae* strain 31R1 resistant to chloramphenicol (20 μg/ml) isolated after plating strain 31R1 on KBCC as above. Bacterial strains were tested for ice nucleation activity by a droplet freezing procedure described previously (39). Forty droplets of a bacterial suspension (about 10^9 cells per milliliter) were placed on paraffin-coated aluminum foil sheets floating on a circulating refrigerated ethanol bath maintained at −5 C. A strain was determined to be non-INA if no drops froze. The presence of diffusible fluorescent compounds was detected by observing fluorescence of colonies on KB irradiated with long wavelength ($\lambda = 366$ nm) ultraviolet light.

Evaluation of in vivo antagonism. Non-INA bacterial antagonists were identified by measuring the reduction of frost injury to corn as compared to untreated controls. Approximately 120 three-leaf stage corn (*Zea mays* L.) plants or 50 30-cm-high potato or tomato plants, one plant per replication, were sprayed with a bacterial suspension (about 10^8 cells per milliliter in distilled water, about 1.0 milliliters per plant) or with water alone and placed in a mist chamber at 24 C for 2 days or 5 min, as specified later. Plants were then sprayed with an aqueous suspension of *P. syringae* strain 31R13C (5.0×10^5 cells per milliliter, about 0.2 milliliters per plant) or with water alone and incubated for an additional 2 days in a mist chamber at about 24 C. Plants were then removed from the mist chamber, allowed to dry for 30 min, placed in a controlled environmental chamber (National Appliance Co., Portland, OR) at 0 C, and cooled at the maximum rate of the chamber (about 0.3 C min^{-1}) to about −4.5 C, and held at this temperature for 20 min. Plants were then warmed to 24 C. All three leaves of every corn plant were rated for frost injury. A leaf was scored as damaged if any frost injury was apparent. The mean number of leaves per plant damaged, analysis of variance, and mean comparisons were calculated using the General Linear Models Procedure in the Statistical Analysis System (SAS Institute, Cary, NC). Damage was expressed as the fraction of leaves that exhibited frost damage in each treatment. Bacterial strains causing the largest significant reduction in frost damage to greenhouse-grown plants were selected for further field testing.

Measurement of bacterial populations and ice nuclei on leaves. Each sample of greenhouse-grown plants consisted of five three-leaf-stage corn plants (cultivar PX20) grown in sterile vermiculite and treated with bacteria as above (total sample about 8 g). Four replicate leaf samples of each treatment were collected immediately before placing plants in the cooling chamber at 0 C. Each sample was placed in 100-ml washing buffer (0.1 M potassium phosphate buffer, 0.1% bacto peptone, Difco [pH 7.0]) and shaken vigorously for 2 hr on a reciprocal shaker or sonicated in an ultrasonic cleaning bath (Bransonic 52) for 5 min. Appropriate serial dilutions of leaf washings were plated on KBC, KBCR, and KBCC. Antagonistic bacterial population size was estimated by counting colonies arising on KBCR after 3 days incubation at 24 C after accounting for the mass of plant tissue sampled and the volume of buffer into which plants were placed. Population size of INA *P. syringae* strain 31R13C was estimated by counting colonies arising on KBCC after 3 days incubation at 24 C. In most samples, 31R13C populations were less than 1% that of antagonistic bacteria. However, when 31R13C populations determined on KBCC were 1% or more of the antagonistic bacterial populations in a sample, the numbers of 31R13C colonies on KBCR were determined by a replica freezing technique described previously (40) and subtracted from the total colonies enumerated on KBCR to estimate antagonistic bacterial population size. Analysis of variance and comparisons of mean population sizes of log-transformed data were made using the General Linear Model procedure in SAS.

Leaf surface ice nuclei active at −9 C were quantified using a droplet freezing procedure similar to that reported previously (39). At least 40 10-μl droplets of washings of each leaf sample, or of appropriate dilutions of leaf washings, were placed on the surface of a paraffin-coated aluminum foil sheet floating on the surface of a refrigerated constant-temperature circulating ethanol bath maintained at −9 C. When ice nucleation was not detected in undiluted leaf washings, the leaf washings were concentrated 40-fold by centrifugation. Forty milliliters of a suspension of leaf washings were filtered through a porous cloth, centrifuged at 30,000 *g* for 30 min, and the pellet resuspended in 1 ml of distilled water and tested for ice nucleation activity at −9 C as before. The cumulative ice nucleus concentration at −9 C (N(T)) was calculated according to Vali (59) from the fraction (F) of unfrozen droplets at −9 C as: $N(T) = \ln(^1/F) \cdot 10^D/V$, where D is the number of 10-fold serial dilutions to which the original leaf washings were subjected.

Measurement of antibiosis in vitro. Bacterial cultures were tested for the production of compounds inhibitory to *P. syringae* 31R1 on KBC, NGA, MM, and LDM. Plates were spotted with individual strains using a sterile toothpick and incubated for 2 days (KBC, NGA) or 5 days (MM, LDM) at 24 C. Each plate was sprayed with an aqueous suspension (about 2×10^8 cells per milliliter) of *P. syringae* 31R1 (except 1.0×10^6 cells per milliliter on LDM) and incubated an additional 12 hr at 24 C. The presence of diffusible siderophores produced by antagonistic bacteria was detected by comparing the inhibition of *E. coli* strain AN193, which does not produce the siderophore enterobactin, and AN194, the enterobactin-producing parental strain. Antibiosis toward *E. coli* strains AN193 and AN194 was studied as above except that antagonists were spotted only on KBC plates and plates were incubated for 8 hr at 37 C after AN193 or AN194 was sprayed on plates. An antagonistic strain was scored positive for siderophore production if strain AN193 was inhibited and strain AN194 was either not inhibited or inhibited less than strain AN193. Bacterial strains were also spotted onto KBC plates; incubated for 2 days at 24 C; sprayed (about 0.2 milliliters per plate) with $FeCl_3$ (2.0 mg/ml), trypsin (2.5 mg/ml), or a mixture of trypsin (2.5 mg/ml) and $FeCl_3$ (2.0 mg/ml); incubated at 42 C for 2 hr; sprayed (approximately 0.1 milliliters per plate) with a suspension (about 2.0×10^8 cells per milliliter) of *P. syringae* 31R1; and incubated at 30 C for 12 hr. The antibiosis of each strain was quantified as the mean distance from the edge of each colony to the point at which *P. syringae* strain 31R1 or *E. coli* strains AN193 or AN194 could be detected. A strain was scored as positive for production of inhibitory compounds if a clear zone of at least 1 mm was observed.

Selection of antibiosis mutants. Bacterial strains were grown at 30 C with vigorous agitation in KB broth to a logarithmic growth phase (about 3×10^8 cells per milliliter). Ethyl methane sulfonate (EMS) was added (50 mg/ml), shaken vigorously to ensure dissolution of the added EMS, and incubated with vigorous agitation 20–50 min at 30 C, depending on the bacterial strain. Cells were washed three times in KB broth, grown for 4 hr at 30 C, dilution plated (about 30 colonies per plate) on KB, and incubated 36 hr at 30 C. Plates were then sprayed with a suspension

(approximately 2×10^8 cells per milliliter) of *P. syringae* strain 31R1 and incubated at 30 C for 12 hr. Bacterial colonies with no or reduced visible zones of inhibition of *P. syringae* strain 31R1 were selected and purified on an appropriate selective medium. The antagonism of mutant strains to *P. syringae* strain 31R1 both in vitro and in vivo was measured as described above.

RESULTS

In vitro antagonism of bacterial strains. Eighty-eight bacterial strains isolated from surfaces of healthy leaves and flowers of seven plant species reduced significantly the frost sensitivity of corn seedlings when applied before those plants were inoculated with INA bacteria; these strains also became established on inoculated field-grown plants, including pear, almond, potato, tomato, and navel orange, and reduced epiphytic populations of INA bacteria and thus the frost sensitivity of these plants (35,36,42–44, Lindow, *unpublished data*). These effective antagonists identified during this screening process were used in the study.

Fifty-one of the 88 bacterial strains antagonistic to *P. syringae* and *E. herbicola* on leaf surfaces produced compounds inhibitory to *P. syringae* on at least one of several culture media tested, whereas 37 strains did not (Table 1). Thirty-eight of the in vitro antagonists and 25 of the strains that did not produce such compounds in vitro were tentatively identified as *Pseudomonas* sp. due to their production of yellow-green diffusible pigments. Forty-nine of the 51 strains producing inhibitory compounds to *P. syringae* in vitro and 36 of the 37 strains that did not produce such compounds produced a detectable siderophore that was not utilized by AN193 (Tables 1 and 2).

TABLE 1. Characteristics and in vitro antibiotic production of bacteria antagonistic to *Pseudomonas syringae* on leaf surfaces

Characteristic tested	Number of strains[a]			
Production of inhibitory compounds against *P. syringae* on KBC, NGA, or MM	+(51)		−(37)	
Fluorescent pigment production	+(38)	−(13)	+(25)	−(12)
Siderophore production[b]	+(38) −(0)	+(11) −(2)	+(25) −(0)+	(11) −(1)
Inhibition of *P. syringae* by				
Iron-regulated antibiotic[c]	(36)	(6)	0	0
Trypsin-sensitive compounds[d]	(3)	(0)	0	0
Other[e]	(13)	(7)	0	0

[a] Total number of strains = 88.
[b] Indicated by sensitivity of *Escherichia coli* strain AN193 but not AN194 to bacterial strain tested on KB.
[c] Test organism inhibition of *P. syringae* 31R1 was eliminated when $FeCl_3$ was sprayed on KB test plate before 31R1.
[d] Test organism inhibition of *P. syringae* 31R1 was eliminated by adding trypsin but not $FeCl_3$ to media before spraying test plates with indicator strain.
[e] Test organism inhibition of *P. syringae* 31R1 was not completely eliminated by adding trypsin or $FeCl_3$ to media before spraying test plates with indicator strain.

Antagonistic bacteria produced several types of compounds inhibitory to *P. syringae*. The in vitro production of iron-regulated antibiotics was identified as a reduction or elimination of antibiosis toward *P. syringae* when $FeCl_3$ was added to the media (Table 2). Eighty-two percent of bacteria antagonistic to *P. syringae* in vitro produced iron-regulated antibiotics, while 6% produced a trypsin-sensitive, but $FeCl_3$-insensitive, compound characteristic of many bacteriocins (Tables 1 and 2). Thirty-nine percent of bacteria antagonistic to *P. syringae* in vitro produced trypsin-insensitive and iron-insensitive compounds inhibitory to *P. syringae* (Tables 1 and 2) and 27% produced more than one type of inhibitory compound (Tables 1 and 2).

Inhibition of *P. syringae* on a synthetic leaf diffusate medium. The magnitude of inhibition of *P. syringae* by antagonistic bacteria on LDM of different nutrient concentrations differed from that on KB and was either proportional, inversely proportional, or independent of the concentration of nutrients in LDM (Table 3). Ten strains not inhibitory to *P. syringae* on KB, such as A544 and A557, were inhibitory to *P. syringae* on LDM containing low but not high concentrations of nutrients (Tables 3 and 4). However, 23 of the strains that did not inhibit *P. syringae* on KB also were not antagonistic on LDM of any nutrient concentration (Table 4). Sixty-seven percent of bacterial strains producing iron-regulated antibiotics and two of the three strains producing trypsin-sensitive compounds inhibitory to *P. syringae* on KB were not inhibitory on LDM or were inhibitory only at the highest nutrient concentration (Table 4). However, 12 of 20 strains producing trypsin-insensitive and iron-insensitive inhibitory compounds on KB were inhibitory to *P. syringae* at all concentrations of nutrients in LDM (Table 4).

Isolation of antibiotic mutants. Mutants of each of 25 bacterial antagonists with different patterns of in vitro antibiosis were readily obtained after EMS mutagenesis. Mutants were obtained from each of the 10 strains listed in Tables 2 and 3 that produced antibiotics against *P. syringae* 31R1 on KB as well as 15 additional strains representative of the frequency of strains producing each of the three types of antibiotics identified in Table 1. When cell cultures of parental strains producing only one antibiotic were treated with EMS to a 90% kill level, antibiosis mutants were obtained at a frequency of about 3×10^{-4} per surviving cell.

Antagonism of *P. syringae* on corn leaves by mutant strains deficient in antibiotic production. Frost injury to corn plants treated with mutant strains of antagonistic bacteria, deficient in production of iron-regulated antibiotics, was not significantly higher than injury to plants treated with the parental antibiotic-producing strain (Table 5). Mutant strain A529-1, deficient in the production of an iron-regulated antibiotic, still produced an iron-insensitive antibiotic and did not differ from the parental strain, A529, in reducing frost injury to corn (Table 5). All parental strains tested reduced frost damage significantly compared to plants

TABLE 2. Characterization of compounds, produced by selected antagonistic bacteria, that inhibit *Pseudomonas syringae* strain 31R1 and *Escherichia coli* strains

Bacterial strain tested	Source plant	Zone of inhibition (mm)[a]					
		P. syringae 31R1				*E. coli*	
		KB	KB + Fe[b]	KB + trypsin[c]	KB + trypsin + Fe[d]	AN193 KB	AN194 KB
A120	potato	10.0	0	9.7	0	13.0	0
A529	pear	17.0	6.3	15.7	6.0	13.0	0
A511	pear	10.0	0	9.7	0	10.0	0
A560	navel orange	8.0	3.0	6.7	0	8.0	0
A537	pear	3.3	3.0	0	0	26.0	0
A552	almond	8.0	8.0	8.3	8.0	3.0	3.0

[a] The mean of three replications for each treatment.
[b] About 0.2 ml $FeCl_3$ solution was applied to each plate 2 hr before strain 31R1.
[c] About 0.2 ml trypsin solution was applied to each plate 2 hr before strain 31R1.
[d] About 0.2 ml trypsin and $FeCl_3$ solutions was applied to each plate 2 hr before strain 31R1.

treated with water alone before inoculation with *P. syringae*.

The in vivo antagonism of parental and derivative bacterial strains was related to their population size on corn leaves. Frost injury to corn treated with mutant strain A510-21 was significantly higher than to corn treated with the parental strain A510 (Table 6). However, the population size of strain A510-21 on corn at the time of freezing was also somewhat lower than its parental strain (Table 6). In contrast, the populations of four mutant strains deficient in production of an iron-regulated antibiotic of isolate A508 did not differ from the parental strain (Table 6), and the frost sensitivity of corn plants treated with two of these mutants did not differ from plants treated with the parental strain (Table 6).

Mutants of strains A513 and A538 were as effective as the appropriate parental strain in reducing frost injury to corn and colonization of plants by *P. syringae* when applied either before or at the same time as *P. syringae* (Table 7). Frost injury and populations of *P. syringae* and bacterial ice nuclei on corn leaves coinoculated with *P. syringae* and mutant or parental antagonistic strains were generally greater than that on those leaves treated with the antagonistic strains 2 days before inoculation with *P. syringae* (Table 7). The population sizes of parental strains and their respective mutants, deficient in iron-regulated antibiotic production, were similar whether applied before or simultaneously with *P. syringae*, however.

Mutants defective in antibiotic production from nearly all antagonistic bacteria tested were as effective as the parental strains in reducing frost damage to corn seedlings. Only all mutants of one strain, A510, were less effective than the parental strain in reducing frost injury to corn (Table 6). However, 22 of 23 mutant strains unable to produce a single inhibitory compound under the conditions tested did not differ from their respective parental strains in reducing frost damage to corn. Similarly, mutants of two strains unable to produce either an iron-regulated antibiotic or an iron-insensitive antibiotic also did not differ from their respective parental strains in reducing frost injury to corn. In total, mutations affecting antibiosis in 24 of 25 strains tested did not confer a loss of ability to exclude *P. syringae* from leaf surfaces.

TABLE 3. Inhibition of *Pseudomonas syringae* strain 31R1 by selected antagonistic bacteria on LDM containing different nutrient concentrations

Bacterial strain tested	Source plant	Zone of inhibition (mm)[a]				
			LDM concentration (%)[b]			
		KB[c]	10	20	50	100
A121	potato	16	20	22	22	25
A97	potato	18	0	0	0	0
A506	pear	5	24	18	0	0
A560	navel orange	8	0	1	4	9
A552	almond	8	16	8	0	0
A99	potato	29	0	0	0	10
A544	tomato	0	6	3	0	0
A557	potato	0	36	28	20	2

[a] The mean of three replications for each treatment.
[b] Percent of nutritive components of full-strength LDM.
[c] Inhibition of *P. syringae* detected on KB in another experiment.

TABLE 4. Dependence of inhibition of *Pseudomonas syringae* strain 31R1 by antagonistic bacteria on concentration of nutrients in a synthetic leaf diffusate medium

Concentration of LDM at which inhibition of strain 31R1 observed	No. of strains producing specific classes of inhibitory compounds[a]			
	Iron-regulated	Trypsin-sensitive	Other	None
Low-nutrient concentrations only[b]	3	0	1	10
All concentrations[c]	11	1	12	3
Full-strength LDM media only	13	2	6	1
No inhibition at any concentration	15	0	1	23
Total strains tested	42	3	20	37

[a] Inhibition of *P. syringae* strain 31R1 grown on KB.
[b] Concentrations of 10 or 20% of the nutritive components of full-strength LDM.
[c] Concentrations of 10, 20, 50, or 100% of the nutritive components of full-strength LDM.

DISCUSSION

Several recent investigations have shown the potential for control of fungal diseases (6,16,17,31,33,45,50,53–55) or bacterial diseases (2–6,8–11,14,15,18–21,25–27,32,49,51,54,57,58,61) or the enhancement of plant growth (29,30) with antagonistic bacteria or other microorganisms. Many examples substantiate the biological control of fungal or bacterial pathogens that has been achieved by applying microorganisms antagonistic to the target pathogen in vitro (4,6,10,17,21,25,31,33,54,58). In many cases, a relationship between antibiotic production in vitro, such as siderophores or bacteriocins, and biological control of plant disease has been suggested (2–4,29,30,58). However, several microorganisms not exhibiting in vitro antagonism against a test pathogen also were shown to be effective biological control agents of that pathogen in the greenhouse or field (14,26,45). Conversely, microorganisms that were highly antagonistic to a plant pathogen in vitro were not antagonistic in greenhouse or field tests (6,9,10,19,31,33,53,54). Therefore, the role of antibiotic production in biological control is unclear.

In this study, many bacteria antagonistic to INA bacteria on leaf surfaces did not produce inhibitory compounds on any culture medium tested. Although no attempt was made to test all strains on an exhaustive list of possible culture media, no evidence of antibiosis was observed on media commonly used for screening

TABLE 5. Activity of antibiosis mutants of antagonistic bacterial strains in reducing frost injury to corn leaves treated with *Pseudomonas syringae*

Strain tested[t]	Zone of inhibition[u] (mm)	Fluorescence[v]	Injury (fraction) of leaves[w]
A6 WT	24	+	0.37 c
A6-5	0	−	0.25 de
A6-10	0	−	0.25 def
A6-16	2	−	0.26 d
A6-11	1	−	0.07 ij
A511 WT	23	+	0.38 c
A511-6	0	−	0.35 c
A511-7	0	−	0.38 c
A97 WT	24	+	0.10 hi
A97-3	0	−	0.14 fgh
A97-2	7	+[z]	0.26 d
A506 WT	18	+	0.38 c
A506-1	7	+[z]	0.47 b
A506-2	7	+[z]	0.36 c
A529 WT	15	+	0.18 efg
A529-1	4	−	0.14 ghi
Water control[x]			0.74 a
Uninoculated control[y]			0.02 j

[t] WT indicates parental antagonist strain. Hyphenated designations refer to the numbered antibiotic-deficient mutant (suffix) of the parental strain (prefix).
[u] Inhibition of *P. syringae* strain 31R1 after 2 days incubation at 24 C on KB.
[v] Production of diffusible yellow-green pigment that fluoresces when irradiated with UV (λ = 366 nm) radiation.
[w] The fraction of leaves injured at −4.5 C. Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[x] Plants sprayed with water only 2 days before inoculation with *P. syringae* strain 31R1.
[y] Plants sprayed with water only at time of inoculation of plants with *P. syringae* strain 31R1.
[z] Indicates a weak fluorescence reaction.

potential antagonists for antibiosis, and many highly effective isolates used in this study would have been excluded based on antibiosis.

Many bacterial strains antagonistic to *P. syringae* on rich culture media did not antagonize *P. syringae* on media resembling the nutrients found on leaf surfaces. Although the number of antagonists tested may be too low to generalize, a lower percentage (26%) of the strains that produced iron-regulated antibiotics on KB were inhibitory to *P. syringae* at all concentrations of LDM, compared with 56% of strains that produced iron-insensitive antibiotics. It is also noteworthy that 64% of strains that were not inhibitory to *P. syringae* on KB or other rich media also were not inhibitory to *P. syringae* on LDM. Thus, the quantity and composition of nutrients in LDM, assumed to be similar to that on leaf surfaces, did not generally induce the production of compounds inhibitory to *P. syringae*. The inhibition of *P. syringae* by some antagonists on LDM may have been by indirect mechanisms such as acid production or nutrient competition in this unbuffered low-nutrient medium, although such factors were not tested. Rapid depletion of nutrients (particularly nitrogen-containing compounds) by antagonists has been indicated as a possible mechanism of antagonism by both bacteria and actinomycetes (6,9,20,25,26). Because many antagonists in this study also were inhibitory to *P. syringae* only on low concentrations of LDM, this may be an important mechanism of interaction of *P. syringae* and other epiphytic bacteria. Similarly, certain antagonistic bacteria rapidly alter the pH of growth media to levels inhibitory to growth of plant pathogens (6,9,20).

In nearly all cases, antibiosis-negative mutants were as effective as parental strains in surviving on leaves and in reducing frost injury caused by *P. syringae* on leaf surfaces. In fact, the leaf surface population size of antagonistic bacteria was much more closely related to reductions in frost damage, populations of *P. syringae*, or numbers of bacterial ice nuclei than was the production of compounds inhibitory to *P. syringae* in vitro. No relationship between magnitude of in vitro antibiosis of the 88 antagonistic strains in this study toward *P. syringae* and reduction of frost injury to corn or other plants was observed. Although the prior establishment of antagonistic bacteria or mutant strains on plants before challenge inoculations of *P. syringae* improved the biological control of bacterially incited frost damage, coinoculation also was effective. Thus, mutants and parental strains did not differ in reduction of frost damage either by preemptive exclusion or when more direct competition was initiated by simultaneous inoculation of plants with antagonists and *P. syringae*. The loss of antibiotic production was not

TABLE 6. Population size and activity of antibiosis mutants of antagonistic bacteria in reducing frost injury to corn leaves treated with *Pseudomonas syringae*

Strain tested[s]	Zone of inhibition[t] (mm)	Fluorescence[u]	Antagonistic bacteria log (cfu/g)[v]	Frost damage (fraction of leaves)[w]
A508 WT	18	+	4.57 a	0.19 f
A508-21	5	+[x]	4.47 a	0.10 ef
A508-20	0	−	4.74 a	0.24 d
A508-22	5	+[x]	4.57 a	0.21 d
A508-23	3	+[x]	4.69 a	0.25 d
A501	18	+	4.38 a	0.17 de
A501-14	2	−	4.52 a	0.09 ef
A501-13	6	+[x]	4.62 a	0.37 c
A510 WT	19	+	4.41 a	0.34 c
A510-21	0	−	4.20 a	0.63 b
Water control[y]			0.69 b	0.76 a
Uninoculated control[y,z]			0.64 b	0.03 f

[s] WT indicates parental antagonistic strain. Hyphenated designations refer to the numbered antibiotic deficient mutant (suffix) of the parental strain (prefix).
[t] Inhibition of *P. syringae* strain 31R1 after 2 days incubation at 24 C on KB.
[u] Production of a diffusible yellow-green pigment that fluoresces when indicated with UV (λ = 366 nm) radiation.
[v] Mean of four replications.
[w] Fraction of leaves injured at −4.5 C. Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[x] Indicates a weak fluorescence reaction.
[y] Plants sprayed only with water 2 days before inoculation with *P. syringae* strain 31R1.
[z] Plants sprayed only with water at time of inoculation of plants with *P. syringae* strain 31R1.

TABLE 7. Epiphytic population size of antibiosis mutants of antagonistic bacterial strains, and numbers of an ice nucleation active *Pseudomonas syringae* strain and ice nuclei on corn leaves, in relation to frost injury to corn seedlings

Antagonist strain tested[r]	Preinoculation with antagonist	Zone of inhibition[s] (mm)	Fluorescence[t]	Bacterial population size[u] (log [cells/g fr. wt.]) Antagonist	*P. syringae* 31R13C	Log (ice nuclei/g)[v]	Frost injury (fraction of leaves)[w]
A538 WT	+	15	−	5.47 e	3.95 ab	1.93 fg	0.50 hi
A538-1	+	0	−	5.46 e	3.33 ab	2.09 fg	0.55 gh
A538-2	+	4	−	5.67 de	3.10 ab	2.39 def	0.48 hi
A513 WT	+	23	+	5.82 cde	2.99 abc	2.86 bcd	0.61 fg
A513-1	+	0	−	6.08 bcd	2.25 bc	1.78 g	0.44 i
A513-3	+	0	−	6.34 ab	2.97 abc	2.00 fg	0.49 hi
A538 WT[x]	−			6.22 bc	3.26 ab	2.45 cdef	0.71 de
A538-1[x]	−			6.20 bc	3.38 ab	2.29 efg	0.79 bc
A538-2[x]	−			5.71 de	3.49 ab	2.47 cdef	0.67 ef
A513 WT[x]	−			6.41 ab	3.18 abc	2.91 bcd	0.73 cde
A513-1[x]	−			6.43 ab	3.97 ab	2.99 bc	0.78 bcd
A513-3[x]	−			6.69 a	4.32 ab	3.18 b	0.82 b
Water control[y]	+			3.03 f	5.03 a	4.42 a	0.96 a
Uninoculated control[y,z]	+			0.00 g	0.60 c	0.02 h	0.01 j

[r] WT indicates parental antagonist strain. Hyphenated designations refer to the numbered antibiotic-deficient mutant (suffix) of the indicated parental strain (prefix).
[s] Inhibition of *P. syringae* strain 31R13C after 2 days incubation at 24 C on KB.
[t] Production of a diffusible yellow-green pigment that fluoresces when irradiated with UV (λ = 366 nm) radiation.
[u] Means followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[v] Ice nucleation activity measured at −9 C.
[w] Fraction of leaves injured at −4.5 C.
[x] Antagonistic bacteria applied only 5 min before application of *P. syringae* strain 31R13C.
[y] Plants sprayed only with water 2 days before inoculation with *P. syringae* strain 31R13C.
[z] Plants sprayed only with water at time of inoculation of plants with *P. syringae* strain 31R13C.

correlated with interactions of mutant antagonists and *P. syringae* on leaf surfaces in most cases. Antibiotic mutants of several plant growth-promoting rhizobacteria also colonized the rhizosphere of treated plants as effectively as parental strains (30) but, unlike the results of this trial, differed from the parental strains in their effects on plant growth (30).

Iron-regulated antibiotics, possibly siderophores, were the most common inhibitory compound produced by antagonistic bacteria in vitro. Siderophores have been implicated in interactions of some plant growth-promoting rhizobacteria on roots (30), and of saprophytic bacteria on leaves (48,56). The iron concentration of banana leaf leachates (65 ng/ml) was observed to be insufficient to repress siderophore production in vitro and was suggested as a limiting factor in the growth of phylloplane microorganisms (48). However, siderophores have not been detected on leaf surfaces. Many mutants in this study selected as deficient in iron-regulated antibiotic production did not differ from parental strains in population size on inoculated leaf surfaces, even in the absence of significant numbers of other microorganisms that might produce siderophores that could be utilized by these mutants. Therefore, the importance of siderophores or other iron-regulated antibiotics in the interaction of saprophytic bacteria and epiphytic INA bacteria is not supported by this study and needs more investigation. Similarly, production of inhibitory compounds by bacteria on leaf surfaces has not been detected and may not be necessary to account for the interactions of epiphytic bacteria reported here. However, more work is required to determine if other mechanisms such as nutrient or site competition or other such competitive exclusion processes are sufficient to account for the effectiveness of bacterial biological frost control agents.

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