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Masking of antibiotic-resistance upon recovery of endophytic bacteria

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Abstract

During studies on internal plant colonization by rhizosphere bacteria and endophytic bacteria over several years, we frequently observed lack of growth of rifampicin-resistant mutants (rif+) on tryptic soy agar amended with rifampicin (RTSA). Following seed treatment of cucumber with 6 species of rif+ rhizosphere bacteria in one experiment, all strains were recoverable on RTSA when external root colonization was monitored. Following trituration of surface-disinfested roots, only one strain grew directly on RTSA; however colonies isolated on tryptic soy agar (TSA) grew within 18 h after transfer to RTSA. We term this temporary loss of the antibiotic-resistant phenotype 'antibiotic masking'. Antibiotic masking was also observed with isolation of 7 rif+ endophytic bacterial strains from inside stems of cotton and with isolation of mutants of bacterial endophytes resistant to polymyxin B sulfate from cotton plants. Rifampicin-masking was not accounted for in vitro by inhibitory compounds from cotton plant extracts, by bacterial growth on low nutrient agar, or by competition with other bacteria. Collectively, these results suggest that expression of antibiotic-resistance may be altered in planta, although causes for this antibiotic-masking remain to be elucidated, methods for quantifying internal plant colonization by rif+ bacteria should account for this possibility.

Introduction

Quite often in ecological studies involving plant-associated bacteria there is a need to inoculate and subsequently recover specific strains from plants. The use of spontaneously generated antibiotic-resistant mutants is a quick and inexpensive method for marking bacteria for recovery (Compeau et al., 1988; Kloepper and Beauchamp, 1992). Antibiotic-resistance has been used for monitoring plant colonization by rhizobacteria (Van Peer et al., 1990), phyllosphere bacteria (Wilson and Lindow, 1992), and *Rhizobium* (Pankhurst, 1977). Mutant recovery is predicated upon resistance to specific antibiotic(s) at specific concentrations present in growth media.

Over the past four years our laboratory has conducted ecological investigations of endophytic bacteria, including monitoring internal plant colonization by rifampicin-resistant (rif+) mutants (Chen et al., 1995). The phenomenon we have termed 'antibiotic-masking' was first observed in our experiments with endophytic bacteria in cotton and cucumber (McInroy et al., 1992). Plants were treated with rif+ mutants, and reisolation from internal plant tissues was attempted 3 - 14 days later after surface-disinfestation of plant tissues. No rif+ mutants were detected with primary isolation on tryptic soy agar amended with rifampicin (RTSA). However, colonies transferred after primary isolation on tryptic soy agar (TSA)(from plants treated with rif+ mutants) to RTSA grew within 18 hours. Controls, consisting of colonies on TSA from nontreated plants, did not grow after transfer to RTSA. The objectives of this study were to confirm the occurrence of rifampicin-masking, determine if antibiotic-masking occurred with mutants to other antibiotics, and to determine if this phenomenon was associated with several experimental parameters, including inhibitors in plant extract, low nutrient availability, microbial competition or the nature of the disinfesting agent. A portion

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of this work was previously published in abstract form (McInroy et al., 1992).

Methods

Confirmation of rifampicin-masking: seed treatment of cucumber

An experiment was designed to calculate mean population densities of six rifampicin-resistant bacteria inside cucumber (*Cucumus sativa*, L) cv. 'SMR-58' roots. Rifampicin-resistant mutants (100 μg mL^{-1}) of six rhizosphere bacteria (89B-27, *Pseudomonas fluorescens*; 89B-47, *Pseudomonas chlororaphis*; 89B-57, *Pseudomonas chlororaphis*; 89B-61, *Pseudomonas fluorescens*; 89B-76 *Pseudomonas chlororaphis*; and 89B-77, *Serratia plymuthica*) were spontaneously generated on tryptic soy agar, (TSA)(Difco, Detroit, Michigan) according to procedures described previously (Kloepper et al., 1980). Rifampicin-resistant mutants were preserved at -80 °C. Bacterial inocula were prepared by harvesting cells from TSA plates amended with 100 μg mL^{-1} rifampicin (RTSA) cultured at 28 °C for 24 h and suspending (approximately 10^{10} cfu mL^{-1}) in 0.02 *M* potassium phosphate buffer, pH 7.0 (PB). Cucumber seeds were dipped into 10.0 mL of bacterial suspensions immediately prior to planting in 10 cm square pots in Promix (Premier Peat, Rivière-du-Loup, Québec, Canada) soilless mix. Controls were dipped into PB. The experimental design was a randomized complete block with seven treatments each replicated six times. Treatments included six rhizobacteria and a nonbacterial control.

At 3, 7 and 14 days after planting, 4 - 6 cm root segments were excised just below the soil line and weighed. Root segments were surface-disinfested with 1.05% sodium hypochlorite (20% household bleach) for 10 minutes, then washed three times in sterile PB prior to trituration with a sterile mortar and pestle in 10.0 mL PB. To check for surface-contamination, 0.1 mL of the third wash for each sample was transferred to 9.9 mL tryptic soy broth (TSB) and incubated at room temperature on a shaker (approximately 200 rpm) or spread-plated onto TSA. After incubation at 28 °C for 3 days, tubes were examined, and tubes with visible growth were not used in calculating means of population densities. This method was previously found to detect identical contamination percentages to placing samples directly in TSB for a few minutes or printing directly on TSA plates. Macerated samples were serially diluted in PB and spiral plated (Spiral Biotech, Bethesda, Maryland) on TSA and RTSA. Colonies which developed on TSA and not on RTSA from primary isolation were transferred to RTSA to confirm rifampicin-resistance. Bacterial colonies were enumerated with a laser colony counter and bacterial enumeration software (Spiral Biotech, Bethesda, Maryland). Population data were transformed into $\log_{10}$ cfu g^{-1} prior to averaging. Counts below the minimum detectable limit ($\log_{10}$ 1.30 cfu g^{-1} fresh weight) were treated as 0 for calculating means.

Confirmation of rifampicin-masking: Stem-injection of cotton

In a separate experiment, greenhouse-grown cotton (*Gossypium hirsutum* L) cv. 'DES 119' plants were stem-injected at the base of the stem with approximately 50 μL of suspensions of rif+ endophytic strains in PB. Suspensions were prepared as described above. The experiment was a randomized block with 8 treatments, each replicated 6 times. Treatments included 7 rif+ mutants (JM-22R1, JM-93R1, JM-147R2, JM-147R3, JM-197R3, JM-198R3 and JM-900R2) and a PB control. Two weeks after inoculation, a 2 - 3 cm long sample of stem was excised just above the site of inoculation and processed as before, with the exception that surface disinfestation was used with 20% hydrogen peroxide for 10 min instead of sodium hypochlorite. Samples were triturated in 10.0 mL PB prior to plating on TSA and RTSA. Colonies recovered on TSA were compared to reference plates of the appropriate parental strain, and numbers of corresponding colonies were enumerated. Population data were transformed into $\log_{10}$ cfu g^{-1} prior to averaging. Counts below the minimum detectable limit ($\log_{10}$ 1.30 cfu g^{-1} fresh weight) were treated as 0 for calculating means. To check that bacteria enumerated in this manner were the rif+ strains, a minimum of 10 colonies from each replication on TSA were transferred to RTSA and incubated for 18 - 24 h.

Antibiotic-masking with other antibiotics

An experiment was conducted to determine if the antibiotic masking which resulted with rifampicin-resistant mutants also occurred with resistance to other antibiotics. Using the methods described above for generating rifampicin-resistant mutants, mutants were generated to the following: tetracycline at 40 μg mL^{-1} (JM-22, JM-193 and JM-900), polymyxin B sulfate at

60 μg mL^{-1} (JM-22, JM-193 and JM-900) and nalidixic acid at 40 μg mL^{-1} (JM-22 and JM-900). Resistance to each antibiotic was confirmed by repeated transfers to TSA amended with the corresponding concentration of antibiotic. Cotton was grown in the greenhouse for 2 weeks prior to inoculation. Suspensions of antibiotic-resistant mutants were prepared as described above and used to inoculate cotton stems by injection. Two weeks later, isolations were conducted from stem tissue as previously described. Sodium hypochlorite, 1.05%, was used as a surface-disinfestant. Sterility checks were conducted as previously described. Growth of mutants on TSA and RTSA was registered as plus/minus. The experiment was a randomized complete block design with 8 treatments (bacterial strains), and 6 replications.

Tests for causes of antibiotic-masking

An experiment was conducted to test the hypothesis that inhibitory compounds in plants caused antibiotic-masking. Leaves were removed from 3 symptom-free cotton plants grown in the greenhouse, and stems were homogenized in a Waring blender with 200 mL dH_2O. The extract was filtered through cheesecloth, No. 2 Whatman's filter paper, and then through a 0.45 micron nylon filter (Corning, Corning, NY). The sterile filtrate, or cotton extract broth (CEB) was used as growth medium for rif+ mutants of 2 endophytes (JM-22R1 and JM-900R2) as was 10% CEB, 50% (TSB) and 10% TSB. Cultures were continuously shaken at room temperature and spiral plated onto three plates each of TSA and RTSA every day for a 10-day period. Cultures were incubated at 28 °C for 48 h and enumerated as above.

We also hypothesized that low-nutrient levels in the plant reduced the capacity of bacteria to grow directly on high-nutrient media amended with rifampicin. This hypothesis was tested in two ways. First, six rif+ strains (JM-22R1, JM-46R1, JM-88R1, JM-247R1 and JM-362R1) were grown on R2A (Difco, Detroit, Michigan), a low-nutrient medium, at 28 °C. Colonies were transferred from the original culture plate to 5.0 mL PB, serially diluted and spiral plated onto three plates each of R2A and to RTSA. Transferral of colonies was conducted each day for 5 days. Cultures were incubated at 28 °C for 48 h and enumerated as above. Secondly, isolations of rif-marked bacteria were made onto 5% TSA and full-strength TSA, both amended with 100 μg mL^{-1} rifampicin. For this experiment, cucumber plants were stem injected as described above with cotton.

To test the hypothesis that rifampicin-masking was associated with microbial competition, four strains, including one rif+ mutant, of endophytic bacteria were grown in combined culture in 10% CEB. Strains used were JM-22R1 (rif+), JM-46, JM-88 and JM-247. Cultures were continuously shaken at room temperature for one week. A 0.1 mL aliquot was spread plated every-other day onto three plates each of TSA and RTSA and incubated at 28 °C for 48 h. Colonies were enumerated as above.

Results

Confirmation of rifampicin-masking: Seed treatment of cucumber

With only one exception, none of the 6 rif+ strains were recovered from surface-disinfested cucumber roots upon primary isolation on RTSA (Table 1). However, 5 strains were recovered by the third sample period (14 days after planting) on TSA, and all were confirmed to be rifampicin-resistant by transfer from TSA to RTSA. Population densities of the recovered endophytes ranged from $\log_{10}$ 1.10 to $\log_{10}$ 4.27 cfu g^{-1}. No bacteria were isolated from control plants on either TSA or RTSA.

Confirmation of rifampicin-masking: Stem-injection of cotton

All of the seven endophytes injected into cotton were recovered from surface-disinfested stems on TSA (Table 2). However, no strains were recovered directly upon primary isolation on RTSA. Four of the seven endophytes grew within 18 h upon transfer from TSA to RTSA. Population densities of the recovered endophytes ranged from $\log_{10}$ 2.99 cfu g^{-1} to $\log_{10}$ 7.53. Noninoculated cotton contained native stem endophytes at the time of sample.

Antibiotic-masking with other antibiotics

Antibiotic-masking was detected with one polymyxin B sulfate-resistant strain, based on failure to isolate directly on TSA amended with polymyxin B sulfate (Table 3). Two other polymyxin B sulfate-resistant strains were recovered from surface-disinfested stems directly onto TSA amended with polymyxin B sulfate,

Table 1. Recovery of rif+ mutants from inside cucumber roots following seed treatment

Strain	Mean $\log_{10}$ cfu g^{-1} fresh root weight[a]								
	3 DAP[b]			7 DAP			14 DAP		
	TSA[c]	RTSA[d]	Chk[e]	TSA	RTSA	Chk	TSA	RTSA	Chk
89B-27	2.51	0[f]	+	3.78	0	+	4.27	0	+
89B-47	0	0		0	0		0	0	
89B-57	0	0		0	0		1.50	0	+
89B-61	0	0		2.26	1.39	+	0	0	
89B-76	0	0		1.39	0	+	1.69	0	+
89B-77	0	0		0	0		1.56	0	+
Control	0	0		0	0		0	0	

[a] Mean of 6 replications.
[b] Days after planting.
[c] Tryptic soy agar.
[d] Tryptic soy agar + 100 μg mL^{-1} rifampicin.
[e] Indicates growth when transferred from TSA to RTSA.
[f] 0 = below minimum detectable limit which was $\log_{10}$ 1.3 cfu g^{-1}.

Table 2. Recovery of rif+ mutants from inside cotton stems following injection

Strain	Mean $\log_{10}$ cfu g^{-1} fresh root weight[a]		
	14 DAI[b]		
	TSA[c]	RTSA[d]	Chk[e]
JM-22R1	7.53	0[f]	7.53
JM-93R1	3.40	0	0
JM-147R2	3.95	0	0
JM-147R3	4.14	0	0
JM-197R3	4.60	0	2.99
JM-198R3	4.44	0	4.44
JM-900R2	3.86	0	3.59
Control	4.44	0	0

[e] Mean of 6 replications.
[b] Days after inoculation.
[c] Tryptic soy agar.
[d] Tryptic soy agar + 100 μg mL^{-1} rifampicin.
[e] Represents the recovered rif-marked population from TSA. Colonies recovered were compared to reference cultures of the appropriate parental strain and were transferred from TSA to RTSA.
[f] 0 = below minimum detectable limit which was $\log_{10}$ 1.3 cfu g^{-1}.

and two nalidixic acid-resistant strains were recovered directly on TSA amended with nalidixic acid. No masking was observed with tetracycline-resistant strains.

Tests for causes of antibiotic-masking

Growth resulted on RTSA plates in all cases when rif+ mutants were grown in CEB, 10% CEB, 50% TSA and 10% TSA (data not shown). Similarly, rif+ mutants grown on a nutrient-poor medium were not affected when transferred to RTSA. Growth occurred on RTSA with no reduction in colony size for any strain when compared to colonies on R2A plates of similar age (data not shown). Microbial competition did not alter recovery of rif+ mutants in vitro. When three strains of endophytic bacteria were grown together with a rif+ mutant (strain JM-22R1) in 10% CEB, attempts to

Table 3. Recovery of antibiotic-resistant mutants from stem-injected cotton

Strain	Resistance	TSa[a]	TSA+Antibiotic[b]	Chk[c]
JM-22	Nalidixic acid (40 μg mL^{-1})	+	+	+
JM-900	Nalidixic acid (40 μg mL^{-1})	+	+	+
JM-22	Polymyxin B sulfate (60 μg mL^{-1})	+	-	+
JM-193	Polymyxin B sulfate (60 μg mL^{-1})	+	+	+
JM-900	Polymyxin B sulfate (60 μg mL^{-1})	+	+	+
JM-22	Tetracycline (40 μg mL^{-1})	+	-	-
JM-193	Tetracycline (40 μg mL^{-1})	+	+	+
JM-900	Tetracycline (40 μg mL^{-1})	+	-	-

[a] Tryptic soy agar.
[b] Tryptic soy agar + appropriate amount of respective antibiotic.
[c] Indicates growth when transferred from TSA to TSA amended with the appropriate amount of the respective antibiotic.

reisolate the mutant on RTSA were successful (data not shown).

Discussion

The results reported here confirm and expand our previous observation that rifampicin-resistant bacteria inside plant tissues may fail to grow upon primary isolation on RTSA, a phenomenon we term, antibiotic-masking. That antibiotic resistance is not truly lost in these cases is demonstrated by growth on RTSA of bacteria first isolated from plants on TSA. Antibiotic-masking is not observed in isolation from external root or leaf surfaces, and hence is only encountered when isolating bacteria from within roots or stems.

It remains unclear what accounts for the observed antibiotic-masking. While we have most frequently encountered masking with rifampicin-resistant mutants, the results presented here indicate that the phenomenon of masking may occur with resistance to other antibiotics. Although neither cotton extracts nor bacterial competition triggered masking in laboratory experiments, it is possible that these factors contribute to masking in planta. Localized host defense reactions may occur in response to endophytic bacterial colonization, and hence, extracts in planta could be of a different chemical constituency than extracts in vitro.

Since the mid 1980's, there has been an increased interest in research on endophytic bacteria (Gagné et al., 1989; Gardner et al., 1984, 1985; Jacobs et al., 1985; Lalande et al., 1989; Lu and Chen, 1989; Rennie et al., 1982). Several isolated endophytic strains have been reintroduced into plants to determine their functional role (Misaghi and Donndelinger, 1990; Van Peer et al., 1990; Whitesides and Spotts, 1991). Two of these studies (Misaghi and Donndelinger, 1990; Van Peer et al., 1990) used rifampicin-marked endophytes to assess colonization, and both estimated populations based on recovery directly onto media containing rifampicin. In both cases, conclusions were reached based on strain-specificity for internal colonization which could have been different if antibiotic-masking occurred. Future work on population dynamics and recovery of introduced endophytic bacteria should be designed to allow for the possibility of antibiotic-masking.

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