Endophytic Bacteria in Symptom-Free Cotton Plants

I. J. Misaghi and C. R. Donndelinger

Department of Plant Pathology, University of Arizona, Tucson 85721.
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ABSTRACT

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Isolates of *Erwinia* sp., *Bacillus* sp., *B. pumilus*, *B. brevis*, *Clavibacter* sp., and *Xanthomonas* sp. were recovered from surface-sterilized radicles, roots, stems, unopened flowers, and bolls of greenhouse- and field-grown cotton plants of cultivars Delta Pine 41 (DP41) and Delta Pine 61 (DP61). These bacteria could not be eliminated from seeds and the above-ground organs by stringent surface sterilization methods. *Erwinia* sp. was the most prevalent bacterium with an average isolation frequency of 69% for DP41 and 51% for DP61. An antibiotic-resistant mutant of *Erwinia* sp. was recovered from stems, flowers, bolls, and roots of DP41 at average reisolation frequencies of 97, 82, 77, and 48%, respectively, after its introduction into germinated seeds. Reisolation frequencies of an antibiotic-resistant *Bacillus* sp. from the above tissues were 34, 12, 5, and 0%, respectively. In contrast, antibiotic-resistant mutants of *B. pumilus*, *B. brevis*, *Clavibacter* sp., and *Xanthomonas* sp. could not be reisolated from these same organs. Similar results were obtained with DP61. The recovered endophytes were not pathogenic to the two cultivars.

Additional keywords: biological control.

Endophytic bacteria have been detected in different tissues of various symptomless plants including cucumber fruits (13,14,20), sugar beet roots (2,3,9), peanut kernels (18), legume roots (7,19), vegetables (20), potato tubers (4,8,21,23), bermuda grass stems (10), and other storage organs (24). A few of the reported endophytic bacteria are known plant pathogens residing within symptomless host or nonhost plants (3). Others have not been known to cause plant disease.

Despite the occurrence of endophytic bacteria in different plants, little is known regarding their identity, diversity, and population levels in different tissues. Endophytic bacteria probably have evolved intimate relationships with their host plants through co-evolutionary processes and may influence plant physiology in ways that have not yet been elucidated. Moreover, their unique ability to survive inside plants with little or no microbial competition makes them potential candidates for biological control. For example, endophytic bacteria may be constructed to carry genes for antibiotics and insecticides against pathogens and insects, respectively (11). Because of their potential importance and usefulness, we studied bacterial endophytes in cotton and developed procedures to verify their endophytic nature.

MATERIALS AND METHODS

Isolation of endophytes. Plant parts were surface sterilized with 0.5 or 1.0% sodium hypochlorite in 0.05% Triton X-100 (a surfactant, Calbiochem-Behring Corp., La Jolla, CA), full-strength (30% commercial preparation) hydrogen peroxide, or 75% ethanol for varying periods to determine the most effective method for eliminating surface contaminants and epiphytes. Tissues were rinsed in sterile water three times and processed as described below.

Acid-delinted seeds of cotton (*Gossypium hirsutum* L. 'Delta Pine 41' ['DP41'] and 'Delta Pine 61' ['DP61']) were washed for 5 min in running tap water and surface sterilized with 1.0% sodium hypochlorite in 0.05% Triton X-100 for 90 min. Seeds were incubated in the dark at 25–27 C in sterile glass petri plates lined with moist filter papers. Ten excised radicles of 3-day-old seedlings were surface sterilized with 0.5% sodium hypochlorite solution plus the surfactant for 2 min, rinsed with sterile distilled water three times, and triturated in a small amount of sterile water with sterilized mortars and pestles. One hundred-microliter aliquots of various dilutions of the extract were spread evenly on King's B (KB) medium (2% proteose peptone No. 3, 0.1% glycerol, 0.15% $MgSO_4$, 0.15% K_2HPO_4, 1.5% agar) and then incubated at 25–27 C for 2–3 days.

Cotton seeds of DP41 and DP61 were surface sterilized with 1.0% sodium hypochlorite in 0.05% Triton X-100 for 90 min and grown on moistened sterile germination paper rolls (12) for 6 days in a growth chamber with three daily cycles of 12 hr of 28–29 C in the light (8,000 lx) and 12 hr of 23–24 C in the dark. The germination paper rolls were sterilized by autoclaving them for 1 hr, allowing them to cool overnight, and autoclaving them again for 1 hr. Two 1-cm-long root sections were removed from each of 10 seedlings and were surface sterilized with 0.5% sodium hypochlorite in 0.05% Triton X-100 for 5 min, rinsed with sterile distilled water, triturated, and cultured for endophytes as described above.

Ten stem sections 4–6 cm long from 1- to 6-wk-old cotton plants of DP41 and DP61 and 10 stem sections 15 cm long from older plants grown in the greenhouse or in the field were surface sterilized by submersion in 0.5% sodium hypochlorite solution containing 0.05% Triton X-100 for 15–30 min depending on the age of the stems. A 1-cm section was cut from the middle of each stem section and rinsed three times with sterile water. Each of 20 1-cm stem sections was cut into 2-mm cross sections, placed in 2 ml of liquid KB shake cultures, and incubated for 3–4 days at 25–27 C, after which 10 μl of the broth was spread over the surface of KB medium. Plates were checked for bacterial colonies after 2–3 days of incubation at 25–27 C. The inner tissue from each of 10 stems of 3- to 4-mo-old field-grown cotton plants also was assayed for endophytes. This was accomplished by ripping apart surface-sterilized stems longitudinally, then transferring 10–20 mg of the tissue to 2 ml of liquid KB medium. After 3–4 days of incubation at 25–27 C, transfers were made to KB medium to recover endophytes as described above.

Unopened flowers and mature as well as immature bolls were collected from field-grown cotton plants of DP41 and DP61 in southern Arizona and examined for insect damage with a dissecting microscope. Those that appeared uninjured were surface sterilized with 0.5% sodium hypochlorite in 0.05% Triton X-100 for 30 min (flowers) or 60 min (bolls). About 100 mg of the inner tissues of bolls and flowers was triturated, diluted, and plated on KB medium as previously described. Between 24 and 52 samples from each plant part of each cultivar were used for isolation.

Identification of bacteria. Colonies from isolation attempts were placed into six groups on the basis of colony morphology, size,

and color. Ten isolates of each group were selected arbitrarily from among colonies recovered from different tissues for identification. To assure uniformity among the selected isolates of each group, each isolate was Gram stained, tested for pectolytic activity (16) and growth at 45 C, and observed for colony characteristics on KB medium, LB medium (1.0% triptone, 0.5% yeast extract, 1.0% sodium chloride, 1.5% agar), and nutrient agar. Two representative isolates from each of the six groups were further characterized with API 20 E and API 50 diagnostic tests (API Analytical Products, Plainview, NY).

Elimination of endophytes from plants. For a meaningful assessment of bacterial population sizes after their introduction into cotton plants, procedures were developed to eliminate native endophytic populations from plants. Hot-water treatment and antibiotic treatment of seeds were used to eliminate or reduce endophytes from seeds. About 200 seeds of DP41 and DP61 were washed under running water for 5 min, placed in a small bag made of nylon screening, and immersed for 1–60 min in 2 L of water maintained at 65, 75, or 85 C. Seeds were cooled immediately by immersing the bag in 2 L of sterile cold water for 4 min (25). In addition, 20 seeds were washed under running water for 5 min, surface sterilized with 0.5% sodium hypochlorite in 0.05% Triton X-100 for 90 min, and germinated on sterile filter paper moistened with either streptomycin or tetracycline solutions ranging from 20 to 100 μg/ml and exposed for 3 days to 25–27 C in the dark. Surface-sterilized seeds germinated in the presence of water served as the control. Radicles of treated and control germinated seeds were assayed for bacterial population sizes according to the procedures described earlier. Heat-treated, antibiotic-treated, and control seeds also were planted in 200-ml styrofoam cups in pasteurized sand-soil mixture (1:3) and placed in the growth chamber under conditions described earlier. The efficacy of the treatments to eliminate endophytes was determined by surface sterilizing petioles from 2-wk-old seedlings grown from treated and control seeds with 0.5% sodium hypochlorite for 15 min and incubating 10 2-mm-thick sections of petioles in 2 ml of liquid KB medium as described above for subsequent dilution plating and bacterial population count.

Introduction of endophytes into plants. Rifampicin-resistant (rif^r) mutants of six endophytes were selected on LB medium supplemented with 120 μg/ml of rifampicin. Selected antibiotic-resistant mutants were tested for their stability by repeated transfers to LB medium without rifampicin followed by transfer to LB medium supplemented with 120 μg/ml of rifampicin. Seeds of DP41 and DP61, surface sterilized with 0.5% sodium hypochlorite in 0.05% Triton X-100 for 90 min, were germinated under sterile conditions in petri plates in the presence of tetracycline solution (60 μg/ml) for 3 days as described above. Germinated seeds were transferred to sterile petri plates containing sterile filter papers moistened with sterile water and allowed to grow for an additional 24 hr before inoculation with rif^r mutants. Surface-sterilized seeds germinated on sterile filter paper moistened with sterile water served as controls. Germinated seeds were inoculated by immersion in bacterial suspensions (10^8 colony-forming units [cfu]/ml) in beakers inside vacuum jars. After 15 min under partial vacuum, the vacuum was released. Germinated seeds infiltrated with suspensions of wild-type endophytic isolates and those infiltrated with sterile water served as controls. Inoculated and control germinated seeds were planted in soil-sand-peat moss mixture (3:1:1, v/v) in 15-cm-wide pots and maintained in a greenhouse at 29–32 C during the day and 26–28 C during the night. Ability of the introduced antibiotic-marked mutants to spread systemically within cotton plants was assessed. This was done by attempting reisolation from surface-sterilized roots, stems, flowers, and bolls up to about 4 mo after planting as previously described except for the use of LB medium supplemented with 120 μg/ml rifampicin. Between 17 and 25 samples from each plant part of each cultivar were used for reisolation.

Antibiotic-resistant mutants and six isolates of each wild-type group also were introduced into plants by injecting small quantities of bacterial suspensions (10^8 cfu/ml) into stems or bolls by hypodermic syringes to determine pathogenicity. Plants were kept in the greenhouse. They were observed for the presence of lesions at the point of inoculation and were compared with the control for abnormalities weekly up to 8 wk after inoculation.

RESULTS

Isolation and identification of endophytic bacteria. Endophytic bacteria were recovered from radicles of 3-day-old germinated seeds and from roots of 6-day-old seedlings aseptically grown from seeds that were surface sterilized with any of the following treatments: 0.5 or 1.0% sodium hypochlorite in 0.05% Triton X-100 for 90 min; 75% ethanol for 5 min, followed by 0.5% sodium hypochlorite in 0.05% Triton X-100 for 1 hr; or 30% hydrogen peroxide for 4 hr. Longer exposure times inhibited seed germination. Endophytes also were recovered from young stems (1 to 3 wk old), old stems (more than 3 wk old to maturity), interior parts of unopened flowers, and mesocarp and endocarp of bolls at different stages of development (Table 1), after these organs were treated with 0.5% sodium hypochlorite in 0.05% Triton X-100 for 15, 30, 30, or 60 min, respectively.

Quantitative data on the relative population sizes of endophytes were obtained only from radicles, 6-day-old roots, and interior parts of flowers and bolls because trituration of woody tissues was not only impractical but also resulted in accumulation of phenolic compounds which prevented bacterial growth. However, qualitative data concerning endophytes in woody tissue were obtained by culturing small pieces of stems in liquid medium followed by dilution plating.

Endophytic bacteria were separated into three groups on the basis of colony morphology on KB medium. Members of group one were gram negative, motile, and formed yellow colonies on KB medium; those in group two were gram positive, nonmotile, with creamy colonies on KB medium; and those in group three were either gram negative or gram positive, motile or nonmotile, and formed light yellow colonies on KB medium. Based on the results from the API 20 E, API 50, and other tests (16,17), members of group one were identified as *Erwinia* sp., those of group two as *Bacillus brevis*, *B. pumilus*, and *Bacillus* sp., and members of group three as *Clavibacter* sp. and *Xanthomonas* sp. Group one (*Erwinia* sp.) was the predominant group isolated. Average isolation frequency (average percent samples from different plant parts from which the bacterium was recovered) was 69% for DP41 and 51% for DP61 (Table 1). Isolation frequency values for group two and group three endophytes were 40 and 13% for DP41 and 22 and 15% for DP61, respectively. *Erwinia* sp. was not only the most prevalent bacterium in almost all of the isolation attempts, but was often the only bacterium recovered from a given tissue. Although the bacterium could be placed in the group of *E. herbicola* based on its yellow pigmentation and the lack of pectolytic activity (6), such assignment did not seem to be appropriate in view of the inadequacy of the present classification schemes for many *Erwinia* species including *E. herbicola* (15).

Elimination of endophytes. *Erwinia* sp., *Bacillus* sp., and *Xanthomonas* sp. were not detectable in 3-day-old germinated seeds and in petioles of 2-wk-old seedlings grown from seeds treated with tetracycline (60 μg/ml). Tetracycline treatments of seeds did not eliminate *B. brevis*, *B. pumilus*, and *Clavibacter* sp. but reduced populations of these endophytes in germinated seeds by 57, 75, and 81% and in petioles by 44, 29, and 73%, respectively. Tetracycline at 60 μg/ml was not phytotoxic to cotton seedlings but was phytotoxic at higher concentrations. Tetracycline at concentrations of 40 mg/ml or lower was not effective. Germination of seeds in the presence of 20 to 60 μg/ml of streptomycin did not result in changes in the population sizes of the endophytes. Higher concentrations of streptomycin were phytotoxic to the germinating seeds. Heat treatment of seeds at 85 C for 10 min did not affect seed germination and resulted in a 49 and 62% reduction in the population size of *Erwinia* sp. in germinated seeds and petioles, respectively. This treatment, however, did not result in any change in the population size of other endophytes. Population sizes of endophytes in germinated seeds and in petioles remained unchanged after heat treatment

of seeds at 65 and 75 C for 20, 30, 40, or 60 min.

Recovery of introduced bacteria. A rifampicin-resistant mutant of *Erwinia* sp. was recovered from roots, stems, flowers, and bolls of both cultivars up to 4 mo after planting of seeds that were infiltrated with this mutant. Of the remaining five species, only a rifampicin-resistant mutant of *Bacillus* sp. was reisolated from stems, flowers, and bolls of DP41 and DP61 with an average isolation frequency of 17 and 6%, respectively (Table 2). Introduction of rif^r endophytic mutants and wild types into the germinated seeds did not result in any visible damage to emerging seedlings and mature plants. Moreover, the dry weights of 5-wk-old seedlings grown from germinated seeds infiltrated with rif^r endophytic mutants were the same as those of control seedlings. Inoculation of plants by injection of stems or bolls with suspensions of rif^r mutants or wild-type isolates did not result in symptoms. It was concluded that the recovered endophytes were not pathogenic to the two cotton cultivars.

DISCUSSION

At least six bacterial species seem to occur as endophytes in the cotton cultivars DP41 and DP61. *Erwinia* sp. was the most frequently isolated endophyte with the highest population level, followed by *Bacillus* sp., *B. brevis*, and *B. pumilus*. *Erwinia* sp. was isolated consistently from all parts of healthy cotton plants and was shown to spread systemically after its introduction into germinated seeds. In addition, endophytes were present in seeds and various tissues throughout the plant during all stages of its

TABLE 1. Isolation frequency, total population, and relative population of endophytic bacteria in different parts of cotton cultivars, Delta Pine 41 (DP41) and Delta Pine 61 (DP61)

Plant part[a]	Cultivar	Number of attempts	Isolation frequency[b] Group[e] 1	2	3	Total population[c] Group 1	2	3	Relative population[d] Group 1	2	3
Radicles	DP41	52	79	60	31	16.6	5.0	1.8	79	16	5
	DP61	48	63	46	19	7.2	4.4	0.7	89	10	1
Roots	DP41	46	87	50	13	10.6	2.4	1.2	73	26	1
	DP61	49	55	22	10	11.1	3.3	0.3	92	6	2
Young stem	DP41	41	56	22	0	ND[f]	ND	ND	ND	ND	ND
	DP61	38	39	11	11	ND	ND	ND	ND	ND	ND
Old stem	DP41	24	38	13	0	ND	ND	ND	ND	ND	ND
	DP61	37	32	11	11	ND	ND	ND	ND	ND	ND
Flowers	DP41	45	67	22	22	10.2	2.9	0.8	32	61	7
	DP61	40	50	13	38	11.8	3.6	0.4	50	42	8
Bolls	DP41	45	84	71	13	8.8	1.8	0.7	90	8	2
	DP61	39	64	28	0	7.3	1.9	0	81	19	0
Average	DP41	44	69	40	13	11.6	3.0	1.1	69	28	4
	DP61	40	51	22	15	9.4	3.3	0.4	78	19	3

[a] Endophytes were isolated from 3-day-old radicles, 6-day-old roots, young stems (1 to 3 wk old), old stems (more than 3 wk old to maturity), and the interior parts of unopened flowers and of mature and immature bolls.
[b] Percent tissue samples from which endophytes were recovered.
[c] Average number of colony-forming units $\times 10^3$/g fresh weight of tissue obtained by dilution plating of triturated tissues.
[d] Relative population size (%) of each group within the total recovered population.
[e] Isolates in group one were identified as *Erwinia* sp., those in group two as *Bacillus* sp., *B. pumilus*, and *B. brevis*, and those in group three as *Clavibacter* sp. and *Xanthomonas* sp.
[f] ND = not determined; populations in stems were not determined because stem tissues could not be triturated completely and because of the toxicity of released oxidized phenolic compounds.

TABLE 2. Reisolation frequency and population sizes of rifampicin-resistant mutants of bacteria in different parts of cotton cultivars, Delta Pine 41 (DP41) and Delta Pine 61 (DP61)

Plant part[a]	Cultivar	Number of attempts	Reisolation frequency[b] *Erwinia* sp.	*Bacillus* sp.	Others	Total population[c] *Erwinia* sp.	*Bacillus* sp.	Others
Young stem	DP41	21	100	33	0	ND[d]	ND	ND
	DP61	25	100	24	0	ND	ND	ND
Old stem	DP41	17	94	35	0	ND	ND	ND
	DP61	20	100	5	0	ND	ND	ND
Flowers	DP41	17	82	12	0	39	2	0
	DP61	19	89	0	0	37	0	0
Bolls	DP41	22	77	5	0	7	1	0
	DP61	18	72	0	0	12	0	0
Roots	DP41	23	48	0	0	11	0	0
	DP61	19	36	0	0	19	0	0
Average	DP41	20	80	17	0	16	1	0
	DP61	20	79	6	0	23	0	0

[a] Rifampicin-resistant mutants of *Erwinia* sp., *Bacillus* sp., *B. brevis*, *B. pumilus*, *Clavibacter* sp., and *Xanthomonas* sp. were introduced into 3-day-old germinated seeds. Germinated seeds were planted in soil, and the ability of the mutants to spread systemically within plants was assessed by attempting reisolation from young stems (1 to 3 wk old), old stems (more than 3 wk, old to maturity), the interior parts of unopened flowers, mature and immature bolls, and 6-day-old roots.
[b] Percent samples from which endophytes were recovered.
[c] Average number of colony-forming units $\times 10^3$/g fresh weight of tissue obtained by dilution plating of triturated tissues.
[d] ND = not determined; populations in stems were not determined because stem tissues could not be triturated completely and because of the toxicity of released phenolic compounds.

development. *Clavibacter xyli* subsp. *cynodontis* also has been shown to spread systemically throughout corn plants after its introduction into the basal stem (22).

In studies of endophytic microorganisms, it is difficult to prove unequivocally that all epiphytes and/or surface contaminants have been eliminated. However, because of the diversity of surface-sterilizing agents employed, the long exposure time, and the consistent recovery of not more than six species of bacteria from surface-sterilized tissues throughout cotton plants, it is likely that these bacteria were endophytes. The strongest evidence for the endophytic nature of the *Erwinia* sp. was provided by reisolation of the bacterium (at a frequency of 80%) from different surface-sterilized parts of plants after introduction of a rif^r mutant of the bacterium into the germinated seeds. The evidence also is relatively strong for *Bacillus* sp. that could be reisolated at a frequency of 12% from cotton tissue after its introduction. Rifampicin-resistant mutants of the other four species could not be recovered from plants after seed inoculation probably because they were not true endophytes. Our inability to recover these mutants also may relate to the inefficiency of the inoculation method or to loss of antibiotic resistance. Average population sizes of naturally occurring endophytes in cotton tissues were relatively small (0.4×10^3 to 11.6×10^3 cfu/g fresh weight, Table 1) and only slightly less than those reported in the root xylem tissue of alfalfa plants (6.0×10^3 to 4.3×10^4 cfu/g fresh weight) (7). Average population sizes of *Erwinia* sp. throughout plants after its introduction into germinated cotton seeds also were relatively low (1.6×10^4 to 2.3×10^4 cfu/g fresh weight, Table 2) compared with levels reported for *C. x. cynodontis* in different tissues of mature corn (1×10^7 to 1×10^9 cfu/g fresh weight) after inoculation of the basal stems (22).

Although the recovered endophytes do not seem to interfere with the growth and development of the two cotton cultivars, they may do so under certain conditions. It has been suggested (14,21) that sudden and drastic environmental and microbiological changes may activate the growth and pathogenic activity of endophytic quiescent pathogens inside symptom-free hosts leading to an outbreak of disease. For example, the development of *Erwinia*-induced cotton boll rot in California (1) after boll injury may be due to activation of a pathogenic endophytic *Erwinia* in the bolls in response to insect damage rather than to the transmission of the bacterium. Population sizes of endophytes also may fluctuate with time. Bugbee et al (2) have shown that the population of endophytic *Corynebacterium sepidonicum* in sugar beet roots increased sixfold after 150 days of storage at 4–6 C.

The potential use of endophytic bacteria for biological control of pathogens and insects deserves attention. A transformed isolate of *C. x. cynodontis* carrying a delta-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* is being tested for its potential as an endophytic biopesticide in corn against European corn borer (5,11).

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