Introduction and Establishment of Strains of *Enterobacter cloacae* in Golf Course Turf for the Biological Control of Dollar Spot

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ABSTRACT

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Topdressings prepared from cornmeal-sand mixtures infested with strains of *Enterobacter cloacae* were used to introduce bacterial antagonists into bentgrass putting greens naturally infested with *Sclerotinia homoeocarpa*. Topdressings fortified with *E. cloacae* strains EcCT-501 and E1 significantly reduced dollar spot disease development when compared with untreated plots. In some experiments, monthly applications of strain EcCT-501 provided up to 63% disease control and was as effective as iprodione or propiconazole in reducing dollar spot severity. Disease suppression induced by strains EcCT-501 and E1 was evident up to 2 mo after application. Dollar spot suppression was more effective when *E. cloacae* was applied as a preventive treatment rather than a curative treatment. However, strain EcCT-501 significantly reduced dollar spot severity when applied to severely diseased turf. Introduced populations of selected strains of *E. cloacae* in putting greens were monitored in both 1988 and 1989. Recoverable populations of selected *E. cloacae* strains immediately after application were approximately 10^7–10^9 colony-forming units (cfu) per gram dry weight of thatch in both 1988 and 1989. Although populations in both years declined, they remained at levels greater than 10^4 cells per gram for up to 13 wk and were detectable in the spring of 1989 after 1988 summer applications.

Biological control is a potentially attractive means of reducing fungicide inputs to highly managed golf course turf. Although few indepth studies on the biological control of turfgrass diseases have been conducted, promising results have been obtained with complex mixtures of microorganisms as well as individual antagonists as tools for managing fungal turfgrass diseases. In laboratory and greenhouse studies, antagonists suppressive to pythium blight caused by *Pythium aphanidermatum* (Edson) Fitzp. (17,24) and take-all patch caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *avenae* (E. M. Turner) Dennis (27,28) have been described. In field studies, applying preparations of *Typhula phacorrhiza* (Reichard:Fr.) Fr. to creeping bentgrass swards provided up to 74% control of gray snow mold caused by *T. incarnata* Fr. and *T. ishikariensis* Imai (2,12). Similarly, isolates of binucleate *Rhizoctonia* spp. and *Laetisaria arvalis* Burdsall have been suppressive to brown patch caused by *Rhizoctonia solani* Kühn on creeping bentgrass putting greens (1,22). Isolates of *Gliocladium virens* J. H. Miller, J. E. Giddens & A. A. Foster have been suppressive to dollar spot on bermudagrass (9). Applications of topdressings amended with composted substrates can also be suppressive to dollar spot and brown patch on creeping bentgrass/annual bluegrass putting greens (15,16).

Dollar spot, caused by *Sclerotinia homoeocarpa* F. T. Bennett, is one of the most common diseases on golf course turf in the United States (19) and was chosen as a model to study the effects of plant-associated bacteria on the severity of turfgrass diseases. The bacterium *Enterobacter cloacae* (Jordan) Hormaeche and Edwards has been shown previously to be an effective biological control agent against a number of plant pathogenic fungi (7,10,13,14,21,25) and to have apparent affinities for many grass species (5,6,11,18). Our objective in this study was to determine whether strains of *E. cloacae* could establish in creeping bentgrass/annual bluegrass golf course putting greens and effectively suppress dollar spot disease development.

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MATERIALS AND METHODS

Culture and maintenance of bacterial strains. Strains of *E. cloacae* used in this study were described previously (13) and have been successfully used as seed treatments for the control of seed- and root-rotting pathogens on other plant species (6,12,13). To prepare *E. cloacae* inoculum, cells were grown on trypticase soy broth (TSB) to mid- to late-log phase at 30 C. Cells were removed from the culture medium by centrifugation (10,000 *g* for 10 min at approximately 4 C) and resuspended in 10 ml of phosphate buffered saline (PBS). Bacterial suspensions were added at the rate of 10 ml/1,300 cm^3 to a cornmeal-sand mixture (1:3:1, cornmeal/sand/water, v/v) that had been previously sterilized by autoclaving (121 C) for 1 hr on each of three consecutive days. After distributing inoculum uniformly by shaking, the cornmeal-sand inoculum was incubated at room temperature (26 ± 2 C) for 24 hr before use in field experiments.

For some experiments, rifampicin-resistant derivatives of selected strains of *E. cloacae* were generated by spreading

1 ml of a suspension (10^{11}–10^{12} cells per milliliter) of mid- to late-log phase cells on trypticase soy agar (TSA) amended with 100 μg of rifampicin per milliliter (TSA-R). Plates were incubated at 30 C for 16–24 hr and developing colonies were transferred to fresh TSA-R. Transfers to TSA-R were repeated four more times before strains were used in experiments. Growth rates of rifampicin-resistant derivatives in TSB were compared with their respective parental strains at 30 C, and only those rifampicin-resistant strains with growth rates similar to the parental types were chosen for use in field studies. Suspensions of these strains were added to the cornmeal-sand inoculum as described earlier.

Field studies. Field plots were established in 1988 and 1989 on creeping bentgrass (*Agrostis palustris* Hudson)/annual bluegrass (*Poa annua* L.) putting greens at the Country Club of Rochester, Rochester, NY, to evaluate various strains of *E. cloacae* as biological control agents for the suppression of dollar spot. Putting greens were more than 60 yr old and constructed from the native alkaline clay loam soil (pH 7.2) in the area. They contained mixtures of bentgrasses and annual bluegrass and were naturally infested with *S. homoeocarpa*. Greens were mowed at a 5-mm cutting height and aerified (0.6-cm-diameter tynes) 1 day before application of treatments. In 1988, greens received the following fertilizer inputs after application of treatments: a granular 18-0-18 fertilizer on 11 May and 8 September at the rate of 146 kg/ha; a liquid 28-8-18 fertilizer on 18 July at the rate of 43 L/ha; and a 6-2-0 formulation (composted sewage sludge) on 17 November at the rate of 976 kg/ha. In 1989, the following fertilizers were applied: a 18-4-10 fertilizer on 12 May and 12 June at the rate of 146 kg/ha, and a liquid 28-8-18 on 19 September at the rate of 86 L/ha.

Preventive dollar spot treatments were applied on 26 May 1988 to 1.4-m^2 plots at the rate of 465 cm^3 of cornmeal-sand inoculum per square meter. Inoculum was distributed by hand as uniformly as possible over the plot area and lightly rubbed in to distribute the inoculum into the turf canopy. Controls consisted of plots to which no *E. cloacae* inoculum was applied. All plots were then watered by applying 0.6 cm of irrigation. Because disease symptoms did not appear until 5–6 wk after application, all plots were evaluated for dollar spot severity 2 mo after application either by counting the number of infection foci per plot or by evaluating disease severity on a scale of 0–10 where 0 = no disease and 10 = 100% of plot area diseased.

In order to assess the efficacy of curative applications of *E. cloacae* in suppressing dollar spot infections, plots established in 1988 were left untreated for 6 wk (from 29 July to 8 September) to allow symptom development to reach high levels in all plots. On 8 September 1988, plots were rated for disease severity and curative treatments were applied at the same rates as for the preventive treatments. The fungicide iprodione was applied at the rate of 0.6 g a.i./m^2 as a fungicide check. Disease severity was evaluated 2 wk after application.

On 25 May 1989, preventive treatments were again applied as described above to a creeping bentgrass/annual bluegrass putting green at the Country Club of Rochester. This green had been used previously for biological control studies in 1988, but plot areas for the 1989 study did not overlap with those from the 1988 study.

In a separate 1989 study on a second putting green, suppression of dollar spot by *E. cloacae* strain EcCT-501 was compared with the fungicide propiconazole. *E. cloacae* inoculum was applied initially on 25 May 1989 to 0.9 × 0.9 m^2 plots at the rate of 500 cm^3/m^2. Treatments were applied at monthly intervals thereafter. Propiconazole was applied at the rate of 173 mg a.i./m^2. Controls consisted of untreated plots. Plots were rated for disease severity at monthly intervals.

Population dynamics of introduced strains of *E. cloacae*. In both years, populations of rifampicin-resistant derivatives of selected parental strains were monitored over the course of the experiment. In order to sample destructively without damaging plot areas, 20 small cores were removed at random from each of the replicate plots with the aid of a 0.5-cm-diameter cork borer. This sampling method provided approximately 25 g of sample for each treatment replicate. Sample cores contained leaves, roots, rhizomes, thatch, and soil. Cores were removed from a depth of about 3 cm and transported to the laboratory and processed within 24 hr after collection. Ten grams of the core samples from each replicate was macerated in 90 ml of sterile distilled water in a blender for 1 min. A duplicate 10-g sample was placed in an oven (50 C for 5 days) for dry weight determinations. A dilution series from each treatment replicate was prepared in PBS and plated on TSA amended with 50 μg/ml of rifampicin. After incubation at 27 C for 16–24 hr, colonies were counted and population levels were expressed as colony-forming units (cfu) per gram dry weight sample.

Experimental design and data analysis. All field experiments were established as a randomized complete block design with five replicates and conducted in both 1988 and 1989. All experiments were analyzed by analysis of variance and means were separated with the Least Significant Difference (LSD) test. In experiments designed to assess population fluctuations of selected *E. cloacae* strains, linear regression analyses were performed.

RESULTS

Suppression of dollar spot with preventive treatments of *E. cloacae*. *E. cloacae* strains E1 and EcCT-501 significantly reduced dollar spot disease development when compared with untreated plots up to 55 and 64 days after application, respectively (Table 1). In 1988, only strain EcCT-501 significantly reduced dollar spot severity 64 days after application when compared with untreated plots. Similarly in 1989, strain EcCT-501 again significantly reduced dollar spot disease development up to 32 days after application, but by 55 days, disease severity did not differ from that in untreated plots. Strain E1, on the other hand, was ineffective up to 64 days after application in 1988 and 32 days after

Table 1. Suppression of dollar spot on a creeping bentgrass/annual bluegrass putting green with strains of *Enterobacter clocae*[a]

	1988		1989		1989	
Strain	Spots per plot[b]	Control[c] (%)	Rating 1[d]	Control (%)	Rating 2[d]	Control (%)
Untreated	41	...	5.0	...	5.0	...
Uninoculated CMS[e]	35	14.6	3.6	28.0	5.2	0.0
EcCT-501	15	63.4	2.8	44.0	5.4	0.0
EcH-1	28	31.7	4.8	4.0	4.0	20.0
E1	31	24.4	4.4	12.0	3.0	40.0
E6	39	4.9	4.0	20.0	4.4	12.0
E1-R6	23	43.9	NT	...	NT	...
E6-R8	38	7.3	NT	...	NT	...
EcH-1-R8	NT[f]	...	3.8	24.0	4.6	8.0
EcCT-501-R3	NT	...	3.8	24.0	4.6	8.0
LSD ($P = 0.05$)	18		1.6		1.9	

[a] Inoculated into a mixture consisting of 25% cornmeal and 75% fine sand. Applied as a topdressing at the rate of 465 cm^3/m^2.
[b] Number of infection centers per plot area. Ratings assessed 64 days after application.
[c] Based on a percentage of the disease severity in untreated plots.
[d] Rating scale: 1 = 10% of plot area diseased; 10 = 100% of plot area necrotic. Rating 1 taken 32 days after application; rating 2 taken 55 days after application.
[e] CMS = cornmeal/sand mixture.
[f] NT = Not tested.

application in 1989. However, in 1989, only plots treated with strain E1 had significantly less dollar spot severity 55 days after application. Rifampicin-resistant derivatives of E1 and EcCT-501 (E1-R6 and EcCT-501-R3, respectively), as well as all other wild-type strains of *E. cloacae*, were ineffective in suppressing dollar spot activity.

In a separate field experiment in 1989, monthly applications of approximately 10^9 recoverable cells per gram dry weight provided levels of dollar spot suppression equivalent to that provided by monthly applications of the fungicide propiconazole (Table 2). This level of suppression persisted for at least 30 days after application.

Suppression of dollar spot with curative treatments of *E. cloacae*. When applied to highly diseased turf (average disease rating of 5.8), only strain EcCT-501 was effective in reducing dollar spot severity 12 days after application (Table 3). This strain was as effective as iprodione in reducing dollar spot severity, whereas all other strains tested had no significant impact on disease development.

Population dynamics of *E. cloacae*. Introduction of strains of *E. cloacae* in cornmeal-sand topdressing mixtures was effective in establishing high populations in turfgrass foliage, thatch, and soil (Fig. 1A–C). For example, in 1988, topdressing applications of *E. cloacae* strains E1-R6 and E6-R8 established initial recoverable populations of 1.5×10^7 and 4.0×10^7 cfu/g dry wt sample, respectively (Fig. 1A). After 10 wk, populations had declined to 2.5×10^6 and 2.5×10^4 cfu/g dry wt sample, respectively. Similarly, upon reapplication of *E. cloacae* strains on 8 September 1988, populations were established at the rate of approximately 10^8–10^9 cfu/g dry wt sample (Fig. 1B). After 12 days, populations had declined to 2.6×10^5 and 2.0×10^5 cfu/g dry wt sample, respectively. In 1989, EcCT-501-R3 and EcH-1-R8 were established at populations of 3.9×10^8 and 2.9×10^8 cfu/g dry wt sample, respectively (Fig. 1C). After 13 wk, recoverable populations had declined to 3.1×10^5 and 3.7×10^4 cfu/g dry wt sample, respectively. In the spring of 1989 (after the September 1988 application), populations of E6-R3 and E1-R6 were recovered at levels between 10^3 and 10^4 cfu/g dry wt sample (*data not shown*).

DISCUSSION

Most reports on the biological control of turfgrass diseases have come from laboratory and greenhouse experiments (8,17,24,27). There are very few published examples of field successes. Nonetheless, Burpee and colleagues (2,12) have demonstrated effective control of *T. incarnata* on creeping bentgrasses with nonpathogenic isolates of *T. phaccorhiza* in the field. Similarly, Burpee and Goulty (1) obtained up to 90% control of *R. solani* on creeping bentgrass with non-

Table 2. Comparison of biological and chemical suppression of dollar spot on creeping bentgrass/annual bluegrass with *Enterobacter cloacae* (EcCT-501) and the fungicide propiconazole

Treatment	Rating 1[a]		Rating 2[a]	
	Spots per plot	Control (%)	Spots per plot	Control (%)
Untreated	3.4	0.0	19.8	0.0
Uninoculated CMS[b]	3.6	0.0	21.0	0.0
Propiconazole[c]	1.4	58.8	0.6	97.0
E. cloacae (EcCT-501)[d]	2.2	35.3	8.6	56.5
LSD ($P = 0.05$)	0.6		2.8	

[a] Rating 1 (26 June 1989) taken 30 days after the first application. Rating 2 (July 19) taken 23 days after the second application.
[b] Cornmeal/sand mixture consisting of 70% fine sand and 30% cornmeal (v/v).
[c] Propiconazole applied at the rate of 174 mg a.i./m^2 as a fungicide check.
[d] Cornmeal/sand preparations of EcCT-501 applied at monthly intervals. Recoverable populations at the time of application were approximately 10^9 cells per gram dry weight of thatch.

Table 3. Control of dollar spot on a creeping bentgrass/annual bluegrass putting green with curative applications of *Enterobacter cloacae* or fungicides

Treatment[a]	Disease rating[b]	Control[c] (%)
Untreated	5.8	0.0
Uninoculated CMS[d]	4.6	11.5
Iprodione[e]	4.8	17.2
EcCT-501	4.0	31.0
EcH-1	4.2	22.2
E1-R6	5.0	0.0
E-1	5.4	9.1
E-6	5.8	0.0
E6-R8	5.8	0.0
LSD ($P = 0.05$)	1.7	

[a] Applied in a topdressing material (30% organic component, 70% fine sand) at the rate of 465 cm^3/m^2 plot. Treatments were applied 8 September 1988.
[b] Rating scale: 0 = healthy; 10 = 100% foliar blight. Rated at 12 days after application.
[c] Determined as a percentage of the mean disease rating of each respective treatment before application.
[d] CMS = cornmeal/sand (30:70, v/v) mixture.
[e] Iprodione applied at the rate of 0.6 g a.i./m^2.

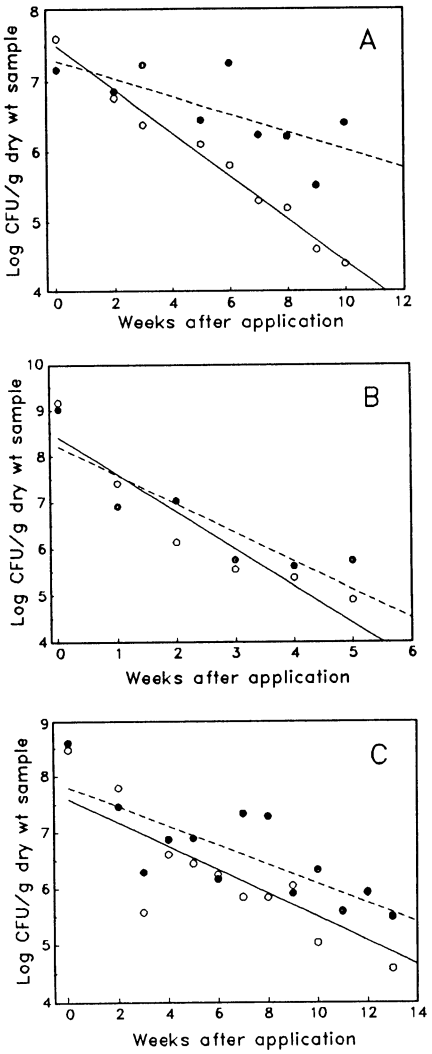

Fig. 1. Population dynamics of introduced strains of *Enterobacter cloacae* in a creeping bentgrass/annual bluegrass putting green. **(A)** Strains E6-R8 (●, dotted line) and E1-R6 (○, solid line) applied 26 May 1988. Regression coefficients: −0.13 ($R^2 = 0.52$) and −0.31 ($R^2 = 0.98$), respectively. **(B)** Reapplication of strains E6-R8 (●, dotted line) and E1-R6 (○, solid line) applied 8 September 1988. Regression coefficients: −0.62 ($R^2 = 0.78$) and −0.42 ($R^2 = 0.96$), respectively. **(C)** Strains EcCT-501-R3 (●, dotted line) and EcH1-R8 (○, solid line) applied 25 May 1989. Regression coefficients: −0.17 ($R^2 = 0.61$) and −0.21 ($R^2 = 0.66$), respectively.

pathogenic binucleate isolates of *Rhizoctonia* spp. Our results have indicated the potential for *E. cloacae* to suppress dollar spot development on creeping bentgrass/annual bluegrass putting greens. The level of control (up to 63%) provided by strain EcCT-501 was as good as curative rates of the fungicide propiconazole for up to 2 mo after application. Furthermore, significant levels of suppression were observed up to 2 mo after application with strains EcCT-501 and E1.

The ability of bacterial biological control agents to establish and survive in association with plants is critical to their performance (4,23). Our results indicate the potential for establishing high populations of bacterial biological control agents in turfgrass ecosystems through topdressing applications on solid substrates. Moreover, the established *E. cloacae* population levels of 10^8–10^9 in our system are quite high in comparison with those reported from the rhizospheres of other graminaceous plants (11). Rifampicin-resistant strains of *E. cloacae* used to monitor populations in turfgrass ecosystems were selected on the basis of in vitro growth rate comparable to the wild type. We have assumed that populations of the wild-type strains behave in a similar manner. However, caution has been raised to the use of this antibiotic marker for monitoring populations of some *Pseudomonas* species in soils (3), because rifampicin-resistant strains of *P. fluorescens* Migula and *P. putida* (Trevisan) Migula are recovered at lower levels than the wild-type strains. Reduced survival is unlikely to result from loss of the mutation because rifampicin resistance is chromosomally encoded. Therefore, if rifampicin-resistant strains of *E. cloacae* are indeed less fit than the wild-type strains in turfgrass systems, our results represent population levels less than populations expected of the wild-type strain. Insofar as we know, however, rifampicin is a suitable marker to follow populations of enteric bacteria in natural soil-plant systems.

The ability of *E. cloacae* to maintain relatively high populations in turfgrass systems may be attributable, in part, to its apparent affinity for members of the Gramineae (5,6,11,18). The ability to efficiently colonize grass plants may be partially responsible for its effectiveness in suppressing dollar spot. Haahtela et al (6) demonstrated that *E. agglomerans* (Beijerinck) Ewing and Fife and related strains of *Klebsiella* spp. could effectively colonize roots of Kentucky bluegrass (*Poa pratensis* L.) and stimulate root hair formation. *Enterobacter* spp. also function in turfgrass rhizospheres as associative nitrogen-fixers. Haahtela et al (6) found that Kentucky bluegrass plants inoculated with some strains of *E. agglomerans* had increased shoot dry matter yields, increased N yields, and increased tissue concentrations of nitrogen as compared with uninoculated plants. The ability of enteric bacteria to provide nitrogen nutrition to turfgrass plants may also, in part, be responsible for disease suppression because dollar spot symptom development on nutrient-deficient turf can be alleviated by fertilizer applications (19).

The mechanisms by which *E. cloacae* suppresses dollar spot disease development are not known. Presumably, the direct interference with pathogen growth and infection involving adherence mechanisms, such as occurs in the interaction of *E. cloacae* with *Pythium* spp. (14), are operative in this system. However, other competitive interactions with pathogen propagules cannot be ruled out (26). While the direct interactions of *E. cloacae* with *S. homoeocarpa* have not been studied, adherence of *E. cloacae* to hyphae of similar fungi such as *S. sclerotiorum* (Lib.) de Bary has been observed in the laboratory (E. B. Nelson, *unpublished data*), suggesting that similar mechanisms may contribute to the control of the dollar spot pathogen.

These biological control studies were conducted on old (>60 yr) soil-based putting greens maintained with a minimum of fungicide inputs. The particular greens used in this study contained an unusually high yet diverse bacterial population (E. B. Nelson, *unpublished data*). While the control of dollar spot by *E. cloacae* on the experimental sites was less than complete (up to 63% suppression), this biocontrol agent performed well among the existing indigenous microflora. It is possible that the performance of *E. cloacae* could be enhanced on putting greens of newer sand-based construction where organic matter and microbial diversity (and, therefore, potential competition) are greatly reduced. Furthermore, sand-based putting greens receiving frequent applications of fungicides capable of altering specific microbial populations (20) might provide an environment less restrictive to the development and biocontrol activity of *E. cloacae*.

The use of topdressing materials fortified with microbial antagonists offers a promising means of establishing populations of antagonists in turfgrass ecosystems and incorporating fungicide alternatives into disease control programs. Golf course superintendents routinely top-dress greens and tees three to four times a season with a mixture of sand and some type of organic matter (usually peat) or soil primarily to smooth the putting surfaces and manage thatch accumulation. The application of antagonist-amended topdressings, therefore, would not introduce additional practices into a turfgrass management program. By introducing biological control agents in this manner, turfgrass managers may be able to convert a relatively inert topdressing material into a biologically active material with fungicidal properties.

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