United States Golf Association Green Section Research Annual Report
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Genetic Basis of Biological Control in a Bacterium Antagonistic to Turfgrass Pathogens

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EXECUTIVE SUMMARY

The main goal of our project on *Enterobacter cloacae* genetics has been to identify the genetic determinants for biocontrol traits in *Enterobacter cloacae* so that their role in the suppression of *Pythium*-incited diseases of turfgrasses can be specifically elucidated. Even though our focus has been on *Pythium*-incited diseases of creeping bentgrass, we believe our studies will have broad applicability to other bacterium-pathogen interactions. The objectives of our studies are to: 1) identify and clone DNA sequences that encode pathogen-suppressive properties in *E. cloacae* strain EcCT-501, 2) determine the nucleotide sequence of *E. cloacae* DNA encoding pathogen-suppressive properties and tentatively establish a function for the gene product, and 3) evaluate, in field studies, the expression of the biocontrol-related genes under typical turfgrass management conditions. Our studies in 1995 focussed primarily on objectives 1 and 2. During work in 1994, we spent considerable time studying mutant V58, which was a biocontrol negative mutant deficient in malate dehydrogenase activity. We further isolated other mutants lacking significant levels of biological control activity. One such mutant, 21-1, was the focus of our studies in 1995. These studies were concerned with establishing the role of fatty acid metabolism in biological control.

The parent strain of *Enterobacter cloacae,* strain EcCT-501, suppresses several different *Pythium* species, including *P. ultimum, P. aphanidermatum,* and *P. graminicola*, on creeping bentgrass. Furthermore, this strain inactivates the stimulatory activity of creeping bentgrass seed exudate, as well as the exudate of many other crop plants, thus preventing responses of these *Pythium* species to plants. With *P. ultimum* in particular, sporangium germination is greatly reduced in the presence of strain EcCT-501. As a result, many of our studies focussed on interactions with *P. ultimum* on creeping bentgrass. From among all exudate components, long chain fatty acids (LCFA) are important stimulants of sporangium germination. Our work in 1995 centered on initial attempts to examine the role of LCFA catabolism in the expression of biological control properties in *E. cloacae*. Strain EcCt-501 reduced the stimulatory activity of the LCFA, linoleic acid, the most abundant LCFA found in creeping bentgrass and other plant seed exudates. A series of Tn*phoA* mutants (Kan^r) were screened for growth on linoleic acid as a sole carbon and energy source. One out of 5000 Kan^r colonies was deficient in the ability to inactivate the stimulatory activity of creeping bentgrass seed exudate and linoleic acid to *P. ultimum* sporangium germination. Furthermore, this mutant, 21-1, no longer protected creeping bentgrass from Pythium seed and seedling disease. A cosmid, pKV1, mobilized into mutant 21-1, complemented the linoleic acid catabolic deficiencies and restored the ability to inactivate creeping bentgrass seed exudate stimulatory activity. Furthermore, this clone fully restored biological control properties to wild-type levels. Current evidence suggests a role of fatty acid metabolism in biological control properties in *Enterobacter cloacae*.

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The main goal of our project on *Enterobacter cloacae* genetics has been to identify the genetic determinants for biocontrol traits in *Enterobacter cloacae* so that their role in the suppression of *Pythium*-incited diseases of turfgrasses can be specifically elucidated. The primary objectives of our studies are to:

1) identify and clone DNA sequences that encode pathogen-suppressive properties in *E. cloacae* strain EcCT-501

2) determine the nucleotide sequence of *E. cloacae* DNA encoding pathogen-suppressive properties and tentatively establish a function for the gene product

3) evaluate, in field studies, the expression of the biocontrol-related genes under typical turfgrass management conditions.

Progress in 1995

The main thrust of our work in 1995 was to further examine biological control mutations in *E. cloacae* strain EcCT-501. Our mutagenesis strategy used in these experiments was identical to that used previously in this project. However, for experiments conducted in 1995, phenotypic screens included seed exudate inactivation assays and linoleic acid inactivation assays along with the standard biocontrol assays. Our reasoning behind the use of these additional screens was as follows: *E. cloacae* is known to protect seeds and seedlings within the first few hours after planting. This is critical since *Pythium* spp. respond extremely rapidly to plant stimuli. Some of our previous work indicated that unsaturated long chain fatty acids (LCFA) were critical molecules for the elicitation of *Pythium* responses to plants and that linoleic acid was the most abundant unsaturated fatty acid found in cotton seed exudate (4) as well as in seed exudate from other plant species, including creeping bentgrass (Penncross) and perennial ryegrass (All*Star) (Nelson and Ruttledge, *unpublished*).

For biocontrol agents to effectively suppress early plant infections by *Pythium* spp., it must express its biocontrol traits rapidly after seeds germinate and plants become established. Competitive interactions between seed-applied bacteria and *Pythium* propagules for critical exudate molecules released during seed imbibition and the subsequent metabolism of these molecules by seed-applied bacteria, could result in reduced propagule germination and seed infection. Our previous research has shown that *E. cloacae* and other seed-applied rhizobacteria can utilize seed exudate from a number of plant species as a sole carbon and energy source and, at the same time, rapidly reduce the stimulatory activity of exudate to *P. ultimum* sporangia (5). Depending on the cell density, this inactivation of 4-hr-

old exudate can occur as rapidly as 2-4 hr.

There is growing evidence that disruption of host-pathogen signalling by seed-applied microorganisms could potentially play an important role in biocontrol processes. Therefore, our efforts focussed on this question relative to the biological control of *Pythium* spp. on creeping bentgrass seedlings. We attempted to determine if the metabolism of unsaturated LCFA can be correlated with the inactivation of seed exudate stimulatory activity and whether this inactivation is related to the biological control of *Pythium* diseases of creeping bentgrass.

Results

Biological Control activity of *E. cloacae* strains.

Enterobacter cloacae strain EcCT-501 was effective in suppressing seedling diseases of creeping bentgrass caused by *Pythium ultimum*, *P. graminicola*, and *P. aphanidermatum* (**Table 1**). Mutants V58 and 21-1 were no longer effective in protecting seedlings from any of the pathogenic *Pythium* species. Although some residual suppression of *P. graminicola was observed with strain* 21-1, this was significantly and substantially different from uninoculated seedlings and is not believed to be a significant level of biological control activity.

Table 1. Differential protection of creeping bentgrass from infection by different *Pythium* species by wild-type, mutant, and complemented strains of *Enterobacter cloacae*.

	Disease Rating (1-5 Scale)		
E. cloacae strain	*P. ultimum*	*P. graminicola*	*P. aphanidermatum*
EcCT-501 (WT)	1.8*	1.3*	2.0*
V-58	4.8	5.0	5.0
21.1	5.0	3.8*	5.0
V-58(pV58K)	2.3*	2.8*	4.3*
21.1(pKV1)	2.0*	2.0*	3.8*
Non-treated	5.0	5.0	5.0
Uninoculated	1.0*	1.0*	1.0*

Means followed by (*) are significantly different from non-treated plants according to T-tests. Rating scale: 1= healthy and 5= 100% unemerged or necrotic. Ratings determined 7 days after inoculation.

Complementing strains V58 and 21-1 with the cosmids pV58K and pKV1, respectively, partially restored biological control activity to that of wild-type levels and levels that did not differ from uninoculated seedlings.

Inactivation of the stimulatory activity of seed exudates and fatty acids by *E. cloacae*.

Growth of *E. cloacae* strain EcCT-501 on either creeping bentgrass seed exudate or on M9 medium containing linoleic acid (0.2 mg/ml) resulted in reduced levels of stimulatory activity after 12 hours of growth (**Figure 1**). Significant reductions in seed exudate stimulatory activity were evident as early as 4 hr after inoculation whereas in M9 media containing linoleic acid, significant reductions in

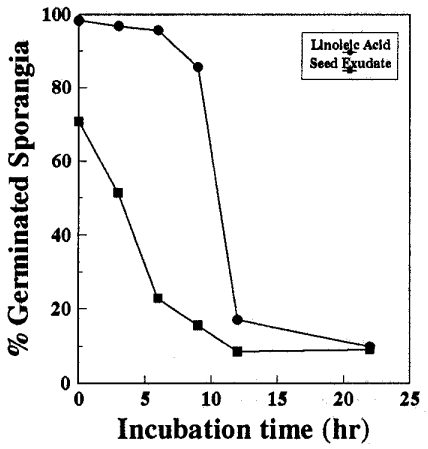

Figure 1. Inactivation of creeping bentgrass seed exudate and linoleic acid solutions by *Enterobacter cloacae* strain EcCT-501. Seed exudate was dissolved in 0.01 M KPO_4 at a concentration of 10 mg/ml whereas linoleic acid was at concentrations of 0.2 mg/ml. Cell-free filtrates were assayed for *Pythium ultimum* sporangium germination.

stimulatory activity were not evident until 9 hr after inoculation. Less than 20% of sporangia germinated in response to either creeping bentgrass seed exudate or M9 containing linoleic acid after 12 to 24 hr incubation with *E. cloacae* EcCT-501. *E. cloacae* strain EcCT-501 was also capable of inactivating the stimulatory activity of several other long chain fatty acids (**Figure 2**). The stimulatory activities of palmitoleic acid, linoleic acid, myristoleic acid, linolenic acid, and eicosadienoic acid were all reduced to less than 5% germinated sporangia after 24 hr incubation with strain EcCT-501.

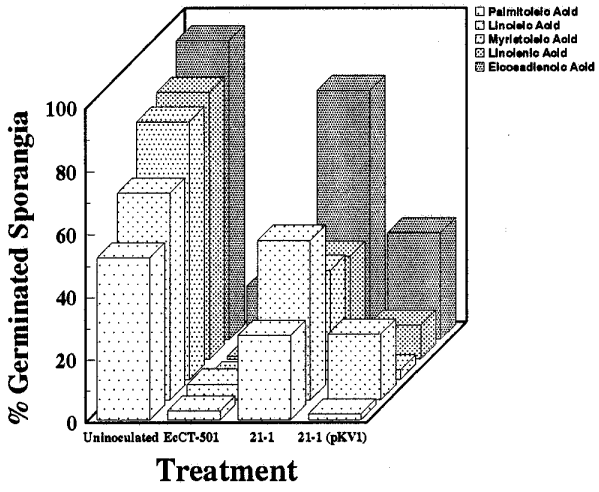

Figure 2. Inactivation of selected fatty acids by *E. cloacae* strain EcCT-501 (wild-type), 21-1 (a biocontrol minus mutant) and 21-1 (pKV1) (complemented mutant with a biocontrol-positive phenotype) after 24 hours growth. Cell-free filtrates were assayed for *Pythium ultimum* sporangium germination.

Analysis of linoleic acid metabolism mutants.

To determine if linoleic acid metabolism was involved in exudate stimulant inactivation, *E. cloacae* EcCT-501R3 was mutagenized using mini-Tn*5phoA* and screened for loss of the ability to grow on linoleic acid as a sole carbon and energy source. Of the approximately 5000 Kan^r transconjugants of *E. cloacae* that were screened, only one, mutant 21-1, was unable to use linoleic acid as a sole carbon and energy source. When strain 21-1 was tested for its ability to inactivate stimulants present in creeping bentgrass seed exudate, high levels of stimulatory activity (60% germinated sporangia) remained after 24 hours of growth when compared to wild-type levels (less then 20% after 6 hours) (**Figure 3**). Furthermore, strain 21-1 was unable to inactivate the stimulatory activity of linoleic acid. After 24 hr incubation of *E. cloacae* strain 21-1 in M9 containing linoleic acid, media were still stimulatory to *P. ultimum* sporangia where nearly 80% germinated (**Figure 4**).

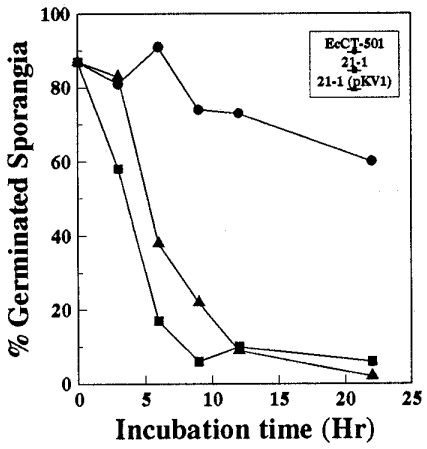

Figure 3. Inactivation of stimulants present in creeping bentgrass seed exudate by *E. cloacae* strains EcCT-501, 21-1, and 21-1 (pKV1). Cell-free exudate filtrates were used to assess germination of *P. ultimum* sporangia.

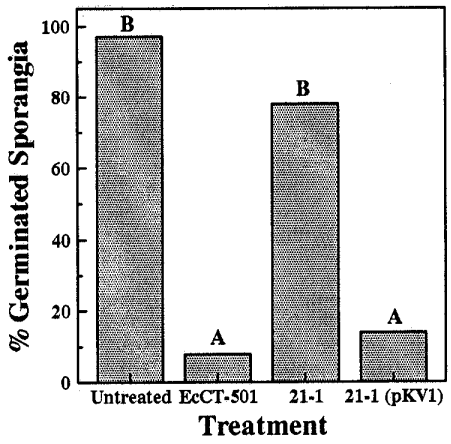

Figure 4. Inactivation of the stimulatory activity of linoleic acid by *E. cloacae* strains EcCT-501, 21-1, and 21-1 (pKV1). Cell-free filtrates were used to assess germination of *P. ultimum* sporangia.

Characterization and complementation of mutant 21-1

The 0.9 kb HindIII internal fragment of mini-Tn*5phoA*, located in the neomycin phosphotransferase gene, was used in Southern analysis to probe a membrane that contained *PstI*-digested total DNA from mutant 21-1 and wild-type. Restriction with *PstI* was chosen because this enzyme cuts the transposon in half, preserving the neomycin phosphotransferase gene which will be used for cloning adjacent DNA. The probe did not hybridize to EcCT-501 DNA, but it did hybridize to a 4.5 kb fragment from mutant 21-1 DNA (data not shown). These results indicate that the 4.5 kb fragment contained part of the Tn*5phoA*.

Several cosmids were capable of restoring the ability of 21-1 to grow on linoleic acid as a sole carbon source. One of these cosmids, pKV1, was used for further studies. pKV1 restored the ability of 21-1 to reduce the stimulatory activity of creeping bentgrass seed exudate to wild-type levels (**Figure 3**). Complementation of 21-1 with pKV1 restored the stimulant inactivation ability to wild-type levels when grown on M9 medium supplemented with linoleic acid and other stimulatory unsaturated fatty acids (**Figure 2 and 4**). Moreover, clone pKV1 restored wild-type levels of *Pythium* seedling disease suppression to 21-1 on creeping bentgrass (**Figure 5**).

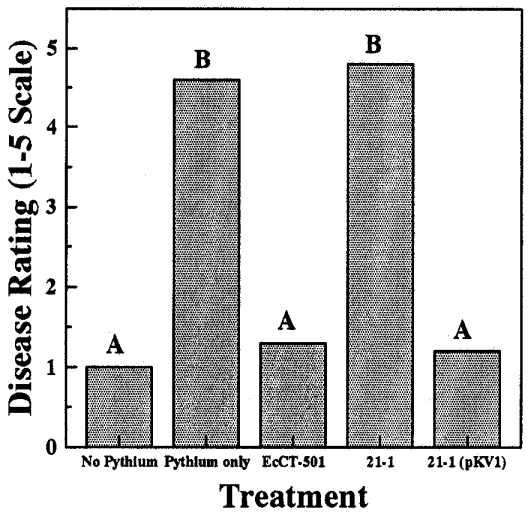

Figure 5. Disease ratings on creeping bentgrass 6 days after inoculation with *Pythium ultimum*. Seedlings treated with *E. cloacae* strains EcCT-501, 21-1, and 21-1 (pKV1). Disease severity rated on a scale of 1-5 for which 1= healthy seedlings and 5= 100% necrotic or unemerged.

Interpretation of Results

The performance of biological control agents has been unreliable and difficult to predict and manipulate, largely because of insufficient knowledge about the mechanisms by which biocontrol microbes interfere with host-pathogen interactions. Most previous studies on mechanisms involved in bacterial biological control of soilborne pathogens have focused on microbe-microbe interactions which result in direct fungal toxicity, such as through the production of antibiotics. This study revealed an important new biocontrol mechanism in which the bacterial biocontrol agent interacts directly with the plant, and only indirectly with the pathogen.

Our previous results have shown that *E. cloacae* is capable of rapidly inactivating the activity of seed exudates stimulatory to *Pythium ultimum* sporangium germination (5), and that linoleic acid is an important stimulatory exudate molecule (4). Results from our present study establish an important role of linoleic acid metabolism by *E. cloacae* strain EcCT-501 in its ability to not only inactivate the stimulatory activity of creeping bentgrass seed exudate, but to suppress *Pythium* infections of seeds. The primary evidence to support this conclusion comes from our studies of *E. cloacae* mutant strain 21-1, a product of mini-Tn*5phoA* mutagenesis of *E. cloacae* EcCT-501. Strain 21-1 could not grow on linoleic acid or inactivate its stimulatory activity, and could not inactivate the stimulatory activity of creeping bentgrass seed exudate to *P. ultimum* sporangia. Furthermore, strain 21-1 could no longer suppress *Pythium* seed rot of cucumber and cotton. All of these phenotypes were fully restored by the cosmid pKV1, carrying a 3.5 kb portion of wild-type DNA. Other molecular genetic studies with *E. cloacae* strain EcCT-501 and mutant derivatives have established similar relationships between exudate stimulant inactivation and *Pythium* seed rot suppression (2)(previous USGA report, 1994). These results establish a strong correlation between linoleic acid metabolism and *Pythium* suppression by *E. cloacae*. However, since pKV1 is not yet characterized, the genetic and biochemical nature of the linoleic acid metabolic deficiency in mutant 21-1 and the mechanism by which *E. cloacae* inactivates fatty acids remains unclear.

At least two explanations for fatty acid stimulant inactivation by *E. cloacae* are possible. First, it is very likely that LCFA uptake and the β-oxidation pathway are centrally involved in removing or reducing the concentration of linoleic acid and possibly other unsaturated fatty acid germination stimulants in seed exudates. By simply removing the fatty acid stimulant from solution through normal uptake mechanisms, the molecule becomes unavailable to *Pythium*. In this case, we predict that genes analogous to *fadL*, *fadD*, and *tsp* in *E. coli*, each of which encodes a specific single receptor/transport protein for LCFA (1, 3), are responsible for initial events in this process. Second, the biohydrogenation of linoleic acid to stearic acid or other saturated derivatives that lack stimulatory activity could result in the reduction or elimination of stimulatory activity in exudate or fatty acid solutions. Our previous work clearly demonstrated that saturated fatty acids contain little or no stimulatory activity (4). Preliminary evidence in our laboratory indicates that the biohydrogenation of linoleic acid to stearic acid by *E. cloacae* strain EcCT-501 could occur (Ruttledge and Nelson, *unpublished*). Biohydrogenation of unsaturated fatty acids has been described for various bacteria, but to our knowledge, this process has not been investigated in bacteria associated with plants under aerobic conditions.

The knowledge that the inactivation of fatty acid germination stimulants could be an important mechanism by which bacterial biocontrol agents interfere with pathogens may have an influence on the screening methods for effective biocontrol organisms, since organisms best capable of inactivating stimulants could be selected. Knowing that by interfering with the release of fatty acids by seeds, seeds might be less susceptible to certain soilborne pathogens, breeding programs for seeds other than those described above can incorporate low fatty acid seed content as a favorable screening criterion.

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