Genetic Basis of Biological Control in a Bacterium Antagonistic to Turfgrass Pathogens

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Goals:

- *Identify and clone genes involved in fatty acid metabolism in E. cloacae strain EcCT-501.*
- *Sequence fatty acid metabolic genes.*
- *Establish relationships between fatty acid metabolism and biological control of Pythium-incited diseases on creeping.*

Cooperator:

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Enterobacter cloacae is an effective biological protectant against infection from many different soil borne plant pathogens. It is particularly effective in suppressing turfgrass diseases incited by *Pythium* species but also is effective against a number of other turfgrass pathogens including *Magneporthe poae* and *Sclerotinia homoeocarpa.*

The precise mechanisms of pathogen and disease suppression by *E. cloacae* are as yet unknown, although a number of traits have been empirically-related to the suppression of seed and seedling rots caused by *Pythium ultimum*. To date, however, no conclusive results point to one major mechanism by which *E. cloacae* suppress diseases caused by *Pythium* species.

An understanding of biological control mechanisms begins with an understanding of the host-pathogen interaction targeted for control. One of the more important aspects of *Pythium* diseases of plants is that these species respond extremely rapidly to germinating seeds and growing roots. *Pythium* spp. are highly dependent on exudate molecules to initiate infection of plants. Microbial interference with the production and activity of such stimulatory molecules could be an effective mechanism of biological control of *Pythium* diseases.

Over the past few years, we have been exploring the possibility of such a mechanism operating with *E. cloacae* and its suppression of *Pythium* diseases. Our previous research with other crop plants demonstrated that *E. cloacae,* and other seed-applied rhizobacteria, can utilize seed exudate from a variety of plant species as a sole carbon and energy source. At the same time, *E. cloacae* rapidly reduced the stimulatory activity of exudate to *P. ultimum* sporangia. Depending on the cell density, this inactivation of exudate can occur as rapidly as 2 to 4 hours.

Other previous work in our laboratory indicated that unsaturated, long-chain fatty acids (LCFA) found in plant exudates were the primary molecules responsible for the elicitation of *Pythium* responses to plants. From analysis of these exudate fatty acids, we found linoleic acid to be the most abundant unsaturated fatty acid found in exudates from a number of plant species, including creeping bentgrass and perennial ryegrass.

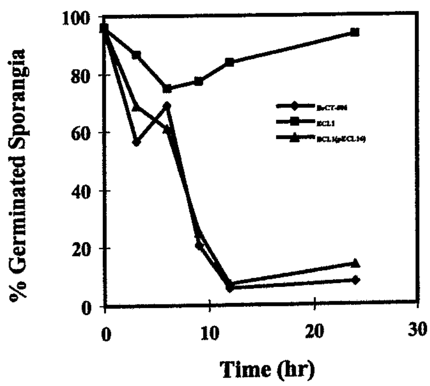
Few bacteria strains recovered from seeds of various plant species reduced the stimulatory activity of perennial ryegrass exudate within 24 hours to levels capable of inducing less than 30 percent *Pythium* sporangium germination. However, of those strains with activity, the majority were strains of *Enterobacter cloacae*. There also was a correlation between the ability of *E. cloacae* strains to inactivate linoleic acid and their ability to protect creeping bentgrass from *Pythium* damping-off.

We have taken a multifaceted approach involving physiological, biochemical, and molecular studies to investigate the relationship between fatty acid metabolism and biological control processes. Wild-type strains of *E. cloacae* can utilize a variety of both unsaturated and saturated LCFA as sole carbon and energy sources but grow very poorly, if at all, on medium-chain length (C_7-C_{11}) fatty acids. In addition, *E. cloacae* is capable of eliminating the stimulatory activity of unsaturated LCFA to *P. ultimum* sporangia in as little as 12 hours.

In our studies, we have used a molecular genetic approach of generating mutants deficient in fatty acid metabolism so that relationships between this function and biological control activity can be observed. We were able to generate a number of mutants of *E. cloacae* strain EcCT-501 that were no longer suppressive to *P. ultimum* in bentgrass assays. It turned out, however, that these were largely mutations in genes encoding TCA (tricarboxylic acid) and DCA (dicarboxylic) cycle enzymes.

More recently, we have been able to generate mutants specifically dysfunctional in the β-oxidation of fatty acids. The first mutant obtained (strain Ec31) fails to grow on media containing linoleic acid as a sole carbon source, but grows well on a minimal media containing succinate. This selection protocol was chosen to avoid selecting mutants with disrupted TCA and DCA cycle enzymes. This mutant is unable to reduce the stimulatory activity of linoleic acid, very slowly reduces the stimulatory activity of seed exudate, and fails to protect bentgrass seedlings from infection by *Pythium* species. Subsequent complementation and sequence analysis has revealed that the mutation in strain Ec31 is in the *fadB* gene, one of five structural genes central to the β-oxidation of fatty acids. While this mutant has debilitated linoleic acid catabolism, it is not clear whether this mutation represents deficiencies in linoleic acid transport or in linoleic acid utilization. Nonetheless, this mutant provides us with a more direct link between the two phenotypes of interest.

As a more direct approach of investigating fatty acid transport in *E. cloacae*, we have focused on the identification and mutagenesis of *fadL* genes within *E. cloacae.* We reasoned that, in such a mutant, we would see a stronger phenotypic response in the presence of linoleic acid. This mutant should allow us to more strongly implicate fatty acid metabolism in the biological control of *Pythium* damping-off of creeping bentgrass. We were successful in obtaining a *fadL* mutant of *E. cloacae* that we designated as strain EcL1. As with the *fadB* mutant (Ec31), the *fadL* mutant (EcL1) was unable to inactivate linoleic acid and seed exudate,



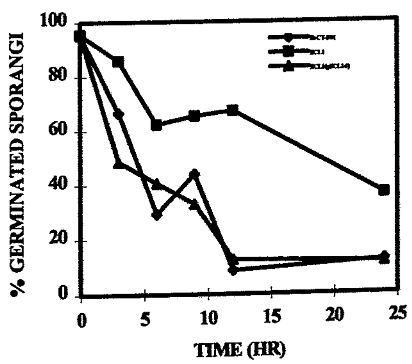


Figure 7. Inactivation of A) linoleic acid and B) seed exudate by E. cloacae strains EcCT-501, EcL1, and EcL1(pECL17).

and unable to protect seedlings from *Pythium* damping-off. Complemented strains fully restored these phenotypes.

Finally, when tested in creeping bentgrass bioassays, the mutant strain EcL1 failed to protect seedlings from damping-off (disease rating 4.5) whereas both the wild-type (EcCT-501) and the complemented strain (EcL1(pECL17)) gave similar and high levels of protection (disease ratings of 1.5 and 1.8, respectively).

Over the course of this project, we have developed strong laboratory evidence for the role of fatty acid metabolism in biological control processes with *Pythium* species on turfgrasses. This represents a novel mechanism of biological control and one that will have important implications for the further development of microbial inoculants and resistant plant germplasm for disease management in bentgrasses.

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 soil borne pathogens have focused on microbe-to-microbe interactions which result in direct fungal toxicity, such as through the production of antibiotics. This study provided convincing evidence for a biological control mechanism in which the bacterial biocontrol agent interacts directly with the plant, and only indirectly with the pathogen.

The inactivation of fatty acid germination stimulants could be an important mechanism by which bacterial biocontrol agents interfere with pathogens. This may have an influence on the screening methods for effective biocontrol organisms, since organisms best capable of inactivating stimulants could be selected. We suspect that the presence of fatty acid metabolizing bacteria such as *E. cloacae* in the spermosphere and rhizosphere of creeping bentgrasses and other grass species may actually impart disease resistance properties to those varieties. Since fatty acids are critical to the developmental biology and pathogenesis of *Pythium* species, it also is likely that breeding varieties for a low fatty acid seed content could be a favorable screening criterion for resistant germplasm. We hope to investigate these hypotheses further in the future.

Table 6. 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*
3-1	4.0	5.0	3.3*
4-1	5.0	5.0	5.0
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 turf and 5 = 100% unemerged or necrotic.
Ratings were determined 7 days after inoculation.