

**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. However, in the last year, we have had to modify our objectives slightly because of recent findings on the nature of pathogen suppression in this system. The current primary objectives of our studies are to 1) identify and clone genes involved in fatty acid metabolism in *E. cloacae* strain EcCT-501; 2) sequence fatty acid metabolic genes; 3) establish relationships between fatty acid metabolism and biological control of *Pythium*-incited diseases on creeping bentgrass.

In 1996, we obtained several additional mutants. The most notable of these are strains 3-1 and 4-1 that fail to grow on media containing linoleic acid as a sole carbon source, but grow well on a minimal media containing succinate. This selection protocol was chosen to avoid selecting mutants with disrupted Krebs cycle enzymes. As with mutants V58 and 21-1, mutants 3-1 and 4-1 are unable to reduce the stimulatory activity of linoleic acid, seed exudate, and to protect bentgrass seedlings from infection by *P. ultimum*. Subsequent complementation and sequence analysis has revealed that the mutation in strain 3-1 is in the *fadAB* operon, which encodes five structural genes central to the β -oxidation of fatty acids. While this mutant is severely debilitated in its ability to catabolize linoleic acid, it is not clear whether this mutation represents deficiencies in linoleic acid transport or in linoleic acid utilization. Therefore, we feel that a search for *fadL* and *fadD* mutants are central to our work.

We have spent considerable effort over the past year trying to sequence the entire *fadAB* operon. We currently have the entire region sequenced upstream of the transposon insertion whereas the downstream portion is nearly 80% sequenced. We are currently in the process of trying to generate *fadL* and *fadD* mutants to allow us to ask questions about the role of fatty acid transport and utilization in biological control processes.

We currently feel we have strong laboratory evidence for the role of fatty acid metabolism in biological control processes with *Pythium* species on turfgrasses. Our work will focus over the next few years in trying to 1) determine whether these processes do indeed function in turfgrass soils; 2) further identify *fadL* and *fadD* mutants that will help us to distinguish between fatty acid uptake and utilization; 3) continue the sequencing of fatty acid genes; and 4) examine turfgrass species and varieties for fatty acid levels in seeds.

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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- 1) identify and clone genes involved in fatty acid metabolism in *E. cloacae* strain EcCT-501
- 2) sequence fatty acid metabolic genes
- 3) establish relationships between fatty acid metabolism and biological control of *Pythium*-incited diseases on creeping bentgrass

Progress in 1996

Background

Our studies over the past several years have focused on the enteric rhizobacterium, *Enterobacter cloacae*, which is widely distributed in nature and, along with other species of *Enterobacter*, is a common component of soil and rhizosphere bacterial communities. Populations of *E. cloacae* are among the more abundant components of seed-associated bacterial communities of many plant species and may constitute one of the predominant associative nitrogen-fixing organisms in the rhizosphere of many cereals and grasses, including turfgrasses.

E. cloacae has been most commonly studied as a biological protectant against infection from seed- and root-infecting fungi on a wide variety of crops and against a number of soilborne plant pathogens. However, it is particularly effective in suppressing diseases incited by *Pythium* species. The precise mechanisms of pathogen 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. Among the more important of these traits is the metabolism of long chain unsaturated fatty acids which are common components of the seed exudate of a variety of plant species including creeping bentgrass and perennial ryegrass.

Our previous research has shown that *E. cloacae* and other seed-applied rhizobacteria can utilize seed exudate from bentgrasses and ryegrasses as a sole carbon and energy source and, at the same time, rapidly reduce the stimulatory activity of exudate to *P. ultimum* sporangia. Depending on the cell density, this inactivation of 4-hr-old exudate can occur as rapidly as 2-4 hr. Furthermore, our research has also shown that

the primary stimulants of sporangium germination in *P. ultimum* consist of long chain unsaturated fatty acids, with linoleic and oleic acids being the most abundant of these in seed exudates.

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.

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 have been trying 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.

Relationship between fatty acid metabolism and biological control

To address the question of the role of unsaturated fatty acid metabolism in biological control processes, we have taken a multifaceted approach involving physiological, biochemical, and molecular studies. We have demonstrated in preliminary experiments that, in the presence of *E. cloacae*, as the stimulatory activity of linoleic acid decreases so too does the concentration of linoleic acid. After 8 hr of growth of *E. cloacae* on linoleic acid solutions, less than 1% of the original amount remains.

To facilitate a search for genes that affect fatty acid metabolism as well as biocontrol phenotypes, we took advantage of the availability of mini-Tn5 constructs to generate mutants. In initial studies, we were able to generate mutants of *Enterobacter cloacae* strain EcCT-501 deficient in biological control phenotypes. However, these were largely TCA cycle mutants. One mutant in particular, V-58, was a malate dehydrogenase mutant. Although not directly involved in fatty acid degradation, the high degree of regulation of fatty acid catabolism in *E. coli* make *mdh* and other dicarboxylic acid enzymes potentially important regulators of fatty acid transport processes.

In an attempt to focus more directly on the mutations in genes affecting fatty acid metabolism, we have screened mutant libraries for the loss of growth on linoleic acid, inability to reduce the stimulatory activity of cotton seed exudate, and for loss of biological control activity. Linoleic acid was chosen since it is the most abundant and stimulatory unsaturated fatty acid found in seed exudates. Wild-type strains of *E. cloacae* can utilize both unsaturated and saturated LCFA as sole carbon and energy sources but grow very poorly, if at all, on medium-chain length (C₇-C₁₁) fatty acids. We have found that, in the presence of linoleic acid, wild-type strains of *E. cloacae* are capable of not only utilizing these fatty acids as sole carbon and energy sources, but of eliminating their stimulatory activity to *P. ultimum* sporangia in as little as 12 hr. We have obtained one mutant (designated as strain 21.1) that lacks the ability to grow on linoleic acid and is incapable of inactivating its stimulatory activity. Furthermore, this strain fails to suppress *Pythium* infections on creeping bentgrass plants (see previous report). Complementation of mutant strains with wild-type sequences restored all previously-disrupted phenotypes.

Fatty Acid Transport and Metabolism in Enteric Bacteria

Fatty acids are readily metabolized by many gram-negative bacteria. These metabolic processes have been well-characterized, with the most significant advances coming from physiological, biochemical, and genetic studies in *Escherichia coli*. The critical first step in the degradation of fatty acids in enteric bacteria is the transport of these molecules into the cell. Three genes, designated *fadL*, *fadD*, and *tsp* encode an outer-membrane protein (FadL), an acyl-CoA-synthetase (FadD), and a periplasmic cotransporter protein (Tsp) involved in transport activation, respectively, that are all involved in the transport of fatty acids to the cytoplasm. FadL is essential for both the binding of exogenous LCFA and its transport into the periplasm. Its expression is induced by the presence of LCFA in the environment. MCFA also can be transported by FadL. However, if FadL is non-functional or uninduced, MCFA can diffuse through the outer membrane. LCFA and MCFA transit the periplasmic space by an as-yet undescribed mechanism but this transport is potentiated by Tsp and may be involved in ligand release from FadL. Fatty acids are transported unidirectionally across the inner membrane and are concomitantly activated to their acyl-CoA thioester derivatives by FadD. This is the terminal step in LCFA and MCFA uptake and the initial step in the β -oxidation pathway, guaranteeing irreversible LCFA and MCFA uptake by the cell.

The β -oxidation pathway has been well studied in *E. coli*, and can be briefly summarized as follows. The utilization of LCFA (C_{12} and greater) by *E. coli* requires the FadR-mediated derepression of the fatty acid transport system described above, and the β -oxidation enzymes. In the oxidation of saturated fatty acids, the enzymes acyl-CoA synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase are required for successive two-carbon shortening of the fatty acid chain, giving rise to acetyl CoA. For unsaturated fatty acids, the additional enzymes 3-hydroxyacyl-CoA epimerase and *cis*- Δ^3 -*trans*- Δ^2 -enoyl-CoA isomerase are required.

The genes encoding all of the β -oxidation enzymes have been cloned and mapped to different sites within the *E. coli* genome. The genes responsible for the transport, acylation, and β -oxidation of LCFA comprise the *fad* regulon, which is induced by LCFA, but not MCFA, even though MCFA serve as substrates for FadD and the other β -oxidation enzymes. Uptake and metabolism of short-chain fatty acid (C_3 - C_6) are controlled by a distinct set of genes that comprise the *ato* regulon. Genes encoding the β -oxidation enzymes, *fadE*, *fadAB*, *fadH*, together with *fadL* and *fadD*, are all transcriptionally regulated by the product of the *fadR* gene (FadR protein). In addition, transcription of the fatty acid biosynthetic gene, *fabA*, is co-regulated by FadR. FadR is a DNA-binding protein that represses the *fad* regulon but activates the *fabA* operon. In the presence of long chain acyl-CoA fatty acid derivatives, FadR releases the *fadAB* and *fadL* promoter, as well as the *fabA* promoters, but with opposite effects: transcription of the *fad* genes is derepressed, while transcription of *fabA* is repressed. The activity of *fadR* is highly repressed by glucose.

Analysis of linoleic acid metabolism mutants.

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The important properties of mutants 3-1 and 4-1 are illustrated in the following figures and in Table 1:

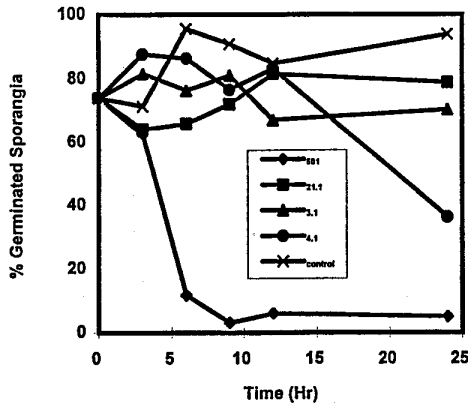


Figure 1. Inactivation of linoleic acid by *E. cloacae* mutants

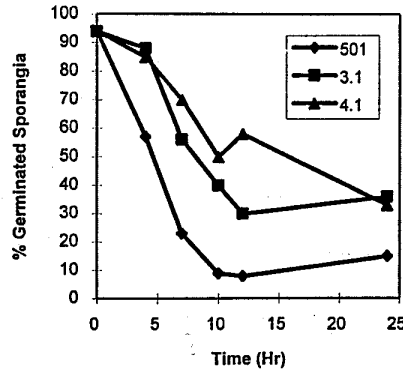


Figure 2. Inactivation of bentgrass seed exudate by *E. cloacae* mutants

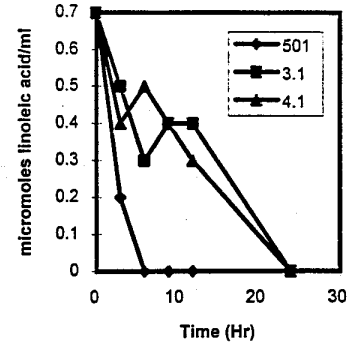


Figure 3. Elimination of linoleic acid by *E. cloacae* mutants

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

<i>E. cloacae</i> strain	Disease Rating (1-5 Scale)		
	<i>P. ultimum</i>	<i>P. graminicola</i>	<i>P. aphanidermatum</i>
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 and 5= 100% unemerged or necrotic. Ratings determined 7 days after inoculation.

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- 1) determine whether these processes do indeed function in turfgrass soils
- 2) further identify *fadL* and *fadD* mutants that will help us to distinguish between fatty acid uptake and utilization
- 3) continue the sequencing of fatty acid genes

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Work Plan if an Additional Years Funds Were Made Available

The work carried out under this project has been conducted by Karin van Dijk, a Ph.D. graduate student. USGA funds have gone to help support the work for her degree. Should an additional year's funding is available, we would plan to continue her support. She plans to graduate in December of 1997. Our work would focus on items 1-4 listed above.