# Fatty Acid Competition as a Mechanism by Which *Enterobacter cloacae* Suppresses *Pythium ultimum* Sporangium Germination and Damping-Off

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Interactions between plant-associated microorganisms play important roles in suppressing plant diseases and enhancing plant growth and development. While competition between plant-associated bacteria and plant pathogens has long been thought to be an important means of suppressing plant diseases microbiologically, unequivocal evidence supporting such a mechanism has been lacking. We present evidence here that competition for plant-derived unsaturated long-chain fatty acids between the biological control bacterium *Enterobacter cloacae* and the seed-rotting oomycete, *Pythium ultimum*, results in disease suppression. Since fatty acids from seeds and roots are required to elicit germination responses of *P. ultimum*, we generated mutants of *E. cloacae* to evaluate the role of *E. cloacae* fatty acid metabolism on the suppression of *Pythium* sporangium germination and subsequent plant infection. Two mutants of *E. cloacae* EcCT-501R3, Ec31 (*fadB*) and EcL1 (*fadL*), were reduced in β-oxidation and fatty acid uptake, respectively. Both strains failed to metabolize linoleic acid, to inactivate the germination-stimulating activity of cottonseed exudate and linoleic acid, and to suppress *Pythium* seed rot in cotton seedling bioassays. Subclones containing *fadBA* or *fadL* complemented each of these phenotypes in Ec31 and EcL1, respectively. These data provide strong evidence for a competitive exclusion mechanism for the biological control of *P. ultimum*-incited seed infections by *E. cloacae* where *E. cloacae* prevents the germination of *P. ultimum* sporangia by the efficient metabolism of fatty acid components of seed exudate and thus prevents seed infections.

In recent years there has been considerable interest in exploiting soil microorganisms for the control of fungal and bacterial plant diseases. A substantial body of research has clearly shown that microorganisms introduced onto subterranean plant parts will colonize seeds and roots, where they provide promising levels of biological disease control of seed- and root-infecting fungal plant pathogens (17). However, the mechanisms of disease suppression are unclear, which has made the successful deployment of microorganisms unpredictable under different environmental conditions.

Current models for microbial control of soilborne plant diseases have focused on antibiotic biosynthesis, parasitism, induced systemic resistance, and microbial competition (57). Whereas there is compelling evidence to support those models involving antibiotic biosynthesis (5), parasitism (24), and induced systemic resistance (54), convincing evidence to support a microbial competition model has been difficult to obtain, in part because of our poor understanding of common critical resources for which introduced bacteria and plant pathogens can compete in rhizosphere and spermosphere habitats.

For competitive interactions to occur, both the introduced microbial strain and the plant pathogen must compete in time and/or space for some common resource. In rhizosphere and spermosphere habitats, limiting concentrations of common critical resources shared by both the pathogen and introduced microbial strains are unknown, and resources utilized by the pathogen during interactions with rhizosphere or spermo-

sphere microorganisms have largely been ignored. We have chosen to study a system in which a common resource is known to be utilized by a plant pathogenic oomycete, *Pythium ultimum*, and the introduced bacterium, *Enterobacter cloacae*.

Our studies focus on *P. ultimum* because it is one of the most destructive plant pathogens, causing seed and root diseases of a wide variety of economically important plants (32). Despite their virulence, *Pythium* spp. are generally considered to be poor microbial competitors in the presence of other plant-associated bacteria (10, 52). To compensate for this, *Pythium* spp. rely on the production of survival propagules such as oospores and sporangia (49, 51), which germinate rapidly in response to fatty acids present in the exudates of germinating seeds (45). This allows *Pythium* species to infect seeds at a time when microbial activities, and thus competitive interactions, are low around the surface of the seed.

An approach for reducing this rapid response to exudates and the subsequent infection of plants by *Pythium* species has been to introduce bacteria on the surface of the seed at the time of planting. One of the more effective bacterial species studied for its *Pythium* suppressiveness is *E. cloacae* (32). A unique feature of some *E. cloacae* strains is that they can rapidly reduce the germination of survival propagules and subsequent seed colonization and infection by *P. ultimum* (53). This reduction in spore germination is expressed within 2 h after a seed is planted (36) and long before radicle emergence.

Although the mechanisms by which *E. cloacae* suppresses *Pythium* seed rot are unknown, we can eliminate mechanisms involving parasitism, antibiosis, or induced systemic resistance. For example, even though *E. cloacae* may attach to the hyphae of *P. ultimum* colonizing seed surfaces, no parasitism is known to occur (38). Unlike many well-studied species of *Pseudomonas* and *Bacillus*, *E. cloacae* does not produce antibiotics or other *Pythium*-inhibitory substances in the presence of seeds or

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TABLE 1. Characteristics of bacterial strain and plasmids used in this study

| Designation | Relevant characteristics | Source or reference |
|---|---|---|
| *Escherichia coli* | | |
|   DH5α | *supE44 ΔlacU169 (f80 lacZΔM15) hsdR16 recA1 endA1 gyrA96 thi-1 relA1*, Nal$^r$ | Life Technologies |
|   LS6164 (or D10) | *fadR ΔfadL* | 14 |
|   LS6164Km2 | *Tn5tac1* insertion in LS6164, Km$^r$ | This study |
|   Sm10λpir | *thi-1 thr leu tonA lacY supE recA*::RP4-2-*Tc*::Mu, Km$^r$ λpir | 34 |
| *Enterobacter cloacae* | | |
|   EcCT-501R3 | Wild type, spontaneous Rif$^r$ | 43 |
|   Ec31 | *fadB*::Tn5*phoA*, Km$^r$ | This study |
|   EcL1 | Δ*fadL*::*nptII* | This study |
| Plasmids and phage | | |
|   pWSK29 | Low-copy-number cloning vector, Amp$^r$ | 55 |
|   pBCKS$^-$ | Cloning vector, Cm$^r$ | Stratagene |
|   pUC19 | Cloning vector, Amp$^r$ | 58 |
|   pBS II SK$^+$ | Cloning vector, Amp$^r$ | Stratagene |
|   PUTKm | Tn5-based delivery plasmid, Km$^r$ Amp$^r$ | 20 |
|   pN103 (or pACC) | Subclone containing *E. coli fadL* in pACYC177, Amp$^r$ | 2 |
|   pRK2073 | A pRK2013 derivative with a TnZ insertion in Km$^r$ gene: mobilizing | 11, 28 |
|   pCPP2277 | Promoterless *uidA* adjacent to *nptII* lacking a transcriptional terminator in pBS II SK$^-$ | D. Bauer, Cornell |
|   pKV311 | PCR clone of *E. cloacae fadBA* in pWSK29, Amp$^r$ | This study |
|   pKVL1 | *fadL* on ~30-kb fragment of EcCT-501R3 genomic DNA on pLAFR3 | This study |
|   pKVL16 | PCR clone of *E. cloacae fadL* in pWSK29, Amp$^r$ | This study |
|   λ::Tn5*tac1* | Tn5 with outwardfacing P*tac* | 4 |

seed exudates (53). Furthermore, suppression of *Pythium* occurs on the surface of the seed coat long before induced systemic resistance mechanisms are known to be expressed in plants (54). Together, these observations suggest a biological control process mediated by competition between *E. cloacae* and *P. ultimum* for specific molecules such as fatty acids released by germinating seeds, which results in the suppression of *Pythium* propagule germination and subsequent plant infection.

The current study was designed to test the hypothesis that competition for seed exudate fatty acids between *E. cloacae* and *P. ultimum* results in the suppression of *Pythium* seed rot. We focused on linoleic acid as a critical and potentially limiting resource since it is necessary for *P. ultimum* to break fungistasis and resume active vegetative growth and since it is also the most abundant stimulatory fatty acid found in cottonseed exudates (45). Although little is known about fatty acid metabolism in *E. cloacae*, much is known about this process in *Escherichia coli* (1), providing a useful model for understanding fatty acid metabolism in *E. cloacae*. Although there are a number of fatty acid degradation (*fad*) genes in *E. coli*, *fadB* and *fadL* genes are among the more important. The gene *fadB* encodes a protein which, together with the product of *fadA*, forms the complex responsible for five of the key β-oxidation enzymes (1), whereas *fadL* encodes an outer membrane protein involved in the binding and transport of fatty acids into the cell (2).

We report here the characterization of the β-oxidation genes *fadB* and *fadL* of *E. cloacae* EcCT-501R3. We provide both genetic and biochemical evidence to support a mechanism for the suppression of a plant disease in which the metabolism of seed exudate fatty acid stimulants of *P. ultimum* sporangium germination by *E. cloacae* reduces sporangium germination and subsequent seed infection. We hypothesize that competition for fatty acids in the spermosphere resulting in the suppression of *Pythium* propagule germination, seed colonization, and seed infection occurs when seed-associated populations of

*E. cloacae* metabolize fatty acids (primarily linoleic acid) released from germinating seeds within this early 12-h window of seed germination.

## MATERIALS AND METHODS

**Microbial strains, plasmids, and culture conditions.** Bacterial strains and plasmids used in this study are described in Table 1. *E. cloacae* strains were routinely grown in Trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.) at 27°C, and *E. coli* strains were grown in Luria-Bertani (LB) medium (33) at 37°C. M9 medium without glucose (33) but supplemented with 25 μg of thiamine HCl/ml is referred to as M9. Sodium linoleate dissolved in Brij58 (Sigma, St. Louis, Mo.) was added to liquid M9 at a concentration of 0.35 mg/ml and to solid media at a concentration of 0.5 mg/ml (M9L). Concentrations of linoleic acid of between 0.2 and 0.5 mg/ml induced 80 to 100% germination of sporangia (Fig. 1).
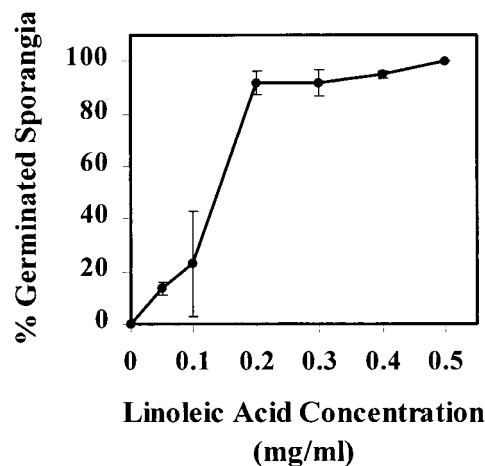


FIG. 1. Dose-response relationships between linoleic acid and *P. ultimum* sporangium germination. Sporangium germination was determined 3 h after exposure to linoleic acid. The error bars represent the standard deviations.

Glucose and succinate were added to a final concentration of 50 mM. Where appropriate, media were supplemented with kanamycin (Km; 50 μg/ml), ampicillin (Amp; 100 μg/ml), rifampycin, (Rif; 100 μg/ml), tetracycline (Tet, 20 μg/ml), chloramphenicol (Cm, 20 μg/ml), and penicillin G (Pen; 50 μg/ml). In media containing linoleic acid or in cottonseed exudate medium, 0.01% butylated hydroxytoluene (BHT; Sigma) was added to reduce fatty acid oxidation.

*P. ultimum* isolate P4 (53) was maintained on wheat leaves in 4 ml of sterile water at 21°C (48). Growth conditions and production of sporangia were as described previously (39, 53). For sporangium germination and seedling bioassay experiments, *P. ultimum* P4 was maintained on solid SM+L medium for 5 days at 24 C. This medium contained the following ingredients per liter: 180 mg of D-glucose, 1.3 mg of L-asparagine, 1 g of soy lecithin (as α-phosphatidylcholine; Sigma catalog number P-5638), 5.3 mg of $(NH_4)_2SO_4$, 2.4 mg of $MgSO_4 \cdot 7H_2O$, 1.1 mg of $CaCl_2$, 3.1 mg of $K_2HPO_4$, 1.6 mg of $KH_2PO_4$, 1.7 μg of thiamine HCl, and 30 g of agar.

**Preparation of cottonseed exudate.** A total of 48 g of cottonseeds (*Gossypium hirsutum* L. Acala SJ-2) was used for all seed exudate batch preparations. Seeds with no cracks or other visible deformations were surface disinfested for 5 min in ~100 ml of 0.5% sodium hypochlorite containing 1 or 2 drops of Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma) as a wetting agent. Seeds were filtered through a sterilized strainer and rinsed three times with sterile water. Surface disinfested seeds were added to 400 ml of sterile water (Ca. one seed/ milliliter) and incubated at 27°C on a rotary shaker at 120 rpm. After 4 h, flask contents were filtered sequentially through two layers of Whatman no. 1 filter paper using 0.8-, 0.45-, and 0.2-μm (pore-size) filters; evaporated under vacuum at 50°C to a volume of 2 to 5 ml; lyophilized; and stored at −20°C. The specific activity of different exudate batches was determined by preparing a series of exudate dilutions in 10 mM potassium phosphate buffer (pH 6.0) and testing for the germination of *P. ultimum* sporangia. The minimum exudate concentration (generally 15 to 20 mg/ml) resulting in 90 to 100% sporangium germination was used in all subsequent experiments.

**DNA manipulations, primers, DNA sequencing, and sequence data analysis.** Standard procedures were followed for DNA manipulations (46). Oligonucleotide synthesis and DNA sequencing were performed at the Cornell Biotechnology Resource Center. DNA sequence data were managed and analyzed using the DNAStar program (DNAStar, Madison, Wis.).

**Mini-Tn5phoA mutagenesis of *E. cloacae* EcCT-501R3 and isolation of a *fadB* mutant.** *E. cloacae* EcCT-501R3 was mutagenized using mini-Tn5phoA as described by de Lorenzo et al. (7). Transconjugants of EcCT-501R3 were tested for growth on linoleic acid as a sole carbon source by replica plating onto solid M9L. Colonies showing little or no growth on M9L were selected and tested for growth on M9 containing 50 mM succinate to ensure that mini-Tn5phoA had not transposed into genes encoding proteins involved in the tricarboxylic or dicarboxylic acid cycles or in oxidative phosphorylation. Transconjugants unable to grow on M9L, but capable of growth on glucose and succinate, were subjected to sequence and complementation analyses to verify their identity as β-oxidation (specifically, *fadBA*) mutants.

**Isolation of an *E. cloacae* *fadL* homolog and construction of an *E. cloacae* *fadL* mutant.** The presence of a *fadL* homolog in *E. cloacae* was confirmed by DNA gel blot analysis by using an internal *EcoRV* fragment of *E. coli* *fadL* as a probe. A pLAFR3 genomic library of *E. cloacae* EcCT-501R3 (30) was mobilized en masse into an *E. coli* *fadL* deletion strain, LS6164Km2, by triparental matings with an *E. coli* strain harboring pRK2073. *E. coli* LS6164Km2 was constructed by introducing a Km marker into *E. coli* LS6164 (FadL⁻ FadR⁻) by infecting LS6164 with λ::Tn5tac1 (4) using procedures described by de Bruijn and Lupski (6). Transconjugants of *E. coli* LS6164Km2 with the *E. cloacae* pLAFR3 library were selected on LB agar containing Km and Tet and transferred to Hybond N membranes (Amersham Life Sciences, Arlington Heights, Ill.) according to the manufacturer's instructions. Membranes were hybridized with a 2.8-kb *EcoRV* fragment of pN103 (2) labeled with $^{32}P$ according to procedures of the Prime-It II random primer kit (Stratagene, La Jolla, Calif.).

One cosmid that hybridized to the *E. coli* *fadL* probe, pKVL1, was used to subclone a 12-kb *HindIII* fragment containing *E. cloacae* *fadL* into the low-copy-number vector pWSK29 to produce pKVL2. *fadL* was disrupted by deletion of an internal 850-kb fragment using unique *EcoRV* and *KpnI* sites followed by the insertion of a *uidA nptII* cassette. This construct was subsequently subcloned into pBCKS⁻ to produce pKLV15. pKVL15 was then used for the homologous recombination of ΔfadL::nptII into the EcCT-501R3 chromosome using the methods described by Roeder and Collmer (44).

**Complementation of *E. cloacae* *fadBA* and *fadL* mutants.** The *fadBA* and *fadL* genes from *E. cloacae* EcCT-501R3 were PCR amplified using standard protocols (23). The primers introduced restriction sites for *XbaI* and *XhoI* to the 5′ and 3′ ends, respectively. Primer sequences were as follows: 5′-end *fadBA* primer, 5′-ATGATCTAGACAGGAGACTGACATGCTC3′; the 3′ *fadBA* primer, 5′-ATGACTCGAGTTACACTCTCTCAAACAC3′; the 5′ *fadL* primer, 5′-ATGATCTAGACTCAATGAGGTTATGGTC-3′, and the 3′ *fadL* primer, 5′-ATGACTCGAGTTAGAACGCGTAGTTGAA-3′. The PCR products were cloned into pWSK29, producing pKV311 and pKVL16 for *fadBA* and *fadL*, respectively. pKV311 and pKVL16 were electroporated into *E. cloacae* strains Ec31 and EcL1, respectively, and tested for their ability to rescue the growth of Ec31 and EcL1 on M9L (16).

**Inactivation of cottonseed exudate and linoleic acid germination stimulating activity by *E. cloacae* strains.** Bacterial cultures were grown overnight in 25 ml of TSB, centrifuged at 5,000 rpm, rinsed, and resuspended in 0.01 M $K_2HPO_4$ (pH 6.0) buffer to $10^9$ CFU/ml as determined by an optical density at 600 nm ($OD_{600}$) of 0.6 for a 1:5 dilution. For growth determinations, 10 μl of the washed cultures were then added to 10 ml of cottonseed exudate (15 mg/ml in 0.01 M $K_2HPO_4$ [pH 6.0]). At 3, 6, 9, 12, and 24 h, the $OD_{600}$ values were determined, and colony counts were obtained by plating aliquots from the cultures onto TSB plates.

For exudate inactivation assays, 0.5 ml of the bacterial suspensions ($10^9$ CFU/ml) were added to 4.5 ml of cottonseed exudate (15 mg/ml in 0.01 M $K_2HPO_4$ [pH 6.0]), incubated in a rotary shaker at 27°C at 100 rpm, and 500-μl portions were removed at intervals, filtered, and frozen at −20°C under an argon atmosphere. These filtrates were assessed for germination-stimulating activity as described previously (53).

For linoleic acid inactivation assays, 2 ml of the bacterial suspensions ($10^9$ CFU/ml) was added to 18 ml of M9L, and the mixtures were incubated at 27°C on a rotary shaker at 100 rpm. At intervals, 700 μl was removed, filtered, and frozen at −20°C under an argon atmosphere. Filtrates were used in sporangium germination assays to determine the germination stimulating activity remaining in liquid M9L and to quantify by gas chromatography the linoleic acid remaining in these solutions (see below).

To determine whether linoleic acid could restore the germination stimulating activity of cottonseed exudate exposed to *E. cloacae* for 24 h, linoleic acid was added at a final concentration of 0.35 mg/ml, and the amended exudate solutions were examined for germination-stimulating activity.

**Gas chromatographic analysis of linoleic acid in culture filtrates.** Linoleic acid was extracted from culture filtrates as described by Folch et al. (12) and esterified at 55°C overnight in 500 μl of methanol containing 2% $H_2SO_4$. Linoleic acid methyl esters were extracted with petroleum ether, dried under a stream of nitrogen, and dissolved in 50 μl of hexane. Then, 0.5-μl injections were made onto a HP5890 gas chromatograph equipped with a 25-m HP-255 GC gas chromatography column (0.2-mm diameter; 0.2-μm film thickness) and a flame ionization detector. The injector temperature was 220°C, and the detector temperature was 230°C. The gas chromatograph was programmed as follows. The initial oven temperature of 180°C was held for 1 min, followed by a 5°C min⁻¹ temperature increase to 220°C, at which point the temperature remained constant for 7 min. The total run time was 17 min. Linoleic acid peak areas and retention times were integrated with a HP3393A integrator. Peak areas were quantified using an external linoleic acid standard. Data were statistically analyzed using pairwise *t* tests.

**Seedling bioassays.** Soil containing inoculum of *P. ultimum* was prepared by mixing 1,600 cm³ of Arkport sandy loam soil, 500 ml of distilled deionized water, and 400 cm³ of sand that had been sieved to a particle size range of 0.5 to 1 mm. This mixture was amended with two macerated petri plate cultures of *P. ultimum* (5 days on SM+L medium; macerated with 100 ml of distilled deionized $H_2O$) and planted repeatedly with 40 surface-disinfested cottonseeds until no seedlings emerged. This replant procedure was used to develop natural stable soil populations of *P. ultimum*. Prior to the bioassay setup, *Pythium* population levels were determined by plating soil dilutions on a *Pythium*-selective medium (35). Noninfested soil was amended with infested soil to adjust the *Pythium* inoculum density to ~$1.2 \times 10^3$ CFU/g of soil. Prior to its use in the bioassays, this soil was mixed with 2 volumes of sand. The resulting mixture had a water content of 3.4%.

Bacterial cultures were grown overnight in 30 ml of TSB, centrifuged at 5,000 rpm, rinsed with 0.02 M $K_2HPO_4$ (pH 7.0), and suspended in 6 ml of 0.02 M $K_2HPO_4$ to a cell density of ~$10^{11}$ CFU/ml.

Bioassays were set up essentially as described previously (53). Glass cylinders (2.2 cm high, 2.2 cm in diameter) were placed on sheets of moist blotter paper positioned on a Plexiglas plate. The paper was marked so that the center of the cylinders were 4 cm apart. *Pythium*-infested soil or noninoculated control soil (3.5 g) was placed in each glass cylinder. One surface-disinfested cottonseed was placed on top of each soil column, drenched with 200 μl of a bacterial cell suspension, covered with 3.5 g of soil, and moistened with 1 ml of $K_2HPO_4$ buffer. There were five cylinders per treatment (used to estimate seedling stands), and the experiments were replicated three times. The entire assembly was placed in a clear plastic box in a 24°C growth chamber with a 16-h photoperiod.

Cylinders were watered daily with distilled deionized water and, after 7 days, were rated for disease incidence and severity. Disease severity was rated on a scale of 0 to 3, where 0 was used to indicate no seedling emergence or an emerged seedling that was completely covered with mycelium; 1 indicated that the emerged seedlings failed to develop and had visible mycelial growth; 2 indicated seedlings that appeared healthy but had visible mycelial growth or seedlings that were less vigorous than those in the noninoculated control cylinders; and 3 represented seedlings that were healthy and had no visible signs of mycelial growth. The disease incidence was calculated as the percentage of remaining healthy seedlings (those receiving a rating of 2 or 3) out of the total seeds planted. Seedling stand data were statistically analyzed using pairwise *t* tests. Disease rating data were analyzed by the nonparametric Mann-Whitney test using pairwise mean comparisons with the aid of Minitab statistical software release 12.1.

## RESULTS

**An *E. cloacae* fadBA mutant, Ec31, is unable to grow on linoleic acid as a sole carbon source.** To test our hypothesis about fatty acid competition between *E. cloacae* and *P. ultimum*, we built upon four important facts. (i) *E. cloacae* protects seeds from *P. ultimum* infection and can reduce the germination stimulating activity of seed exudates to *P. ultimum* sporangia (53). (ii) Sporangia of *P. ultimum* require fatty acids for germination (45) at concentrations of ≥0.2 mg/ml. (iii) Seed infection does not occur without the germination of sporangia (37), which occurs within 1.5 h of being exposed to germinating seeds (50). (iv) The first 12 h of seed germination represents the time frame during which competitive interactions must occur since removal of fatty-acid-containing exudates released within this time period greatly reduces or eliminates seed infections of plants subsequently exposed to *P. ultimum* (15, 18, 38, 40).

We therefore constructed a mutant of *E. cloacae* EcCT-501R3, Ec31, which failed to grow on linoleic acid but which retained the ability to grow on succinate. We reasoned that strains unable to take up or degrade fatty acids should not limit the availability of seed exudate fatty acids to *P. ultimum* for stimulation of sporangium germination and subsequent mycelial growth and would thus not reduce seed infection. Southern analysis revealed a single Tn*5phoA* insertion into the *E. cloacae* genome. Sequence analysis revealed that the disrupted gene encodes a deduced product of 729 amino acids with 92.7% amino acid identity with *E. coli fadB*. Immediately downstream of the *fadB* homolog was a second open reading frame with a deduced product of 365 amino acids and 94.6% identity with *E. coli fadA*. Similar to *E. coli*, the *E. cloacae fadA* and *fadB* genes are in a two-gene operon, with *fadB* transcribed first. A PCR clone of *fadBA* from *E. cloacae*, pKV311, restored the ability of Ec31 to grow on M9L (data not shown).

***E. cloacae* Ec31 cannot inactivate the germination-stimulating activity of linoleic acid and has a reduced ability to inactivate the germination-stimulating activity of cottonseed exudate.** Ec31 was grown in M9L to determine if the *fadB* mutation affected the ability of Ec31 to inactivate the germination-stimulating activity of this growth medium. Even after 24 h of incubation in M9L, Ec31 failed to reduce the germination-stimulating activity of linoleic acid (culture filtrates induced 80 to 90% sporangium germination) (Fig. 2A). In addition to its inability to inactivate the germination-stimulating activity of linoleic acid, Ec31 also failed to inactivate the activity of other long-chain fatty acids, such as myristoleic, palmitoleic, oleic, and linolenic acids (data not shown). However, culture filtrates from EcCT-501R3 and Ec31(pKV311) reduced the germination-stimulating activity such that, within 9 h of growth, the remaining germination-stimulating activity induced only 15 to 20% sporangium germination.

Gas chromatographic analysis of culture filtrates confirmed that Ec31 failed to metabolize linoleic acid. After 12 h of incubation on this medium, 0.32 mg/ml remained in the Ec31 culture, whereas no linoleic acid could be detected in culture filtrates after 12 h of growth of EcCT-501R3 or Ec31(pKV311) (Table 2).

Given that the stimulatory activity of cottonseed exudate is due largely to the presence of fatty acids (45), we tested Ec31 further to determine whether the disruption of β-oxidation in *E. cloacae* had any effect on the bacterium's ability to inactivate
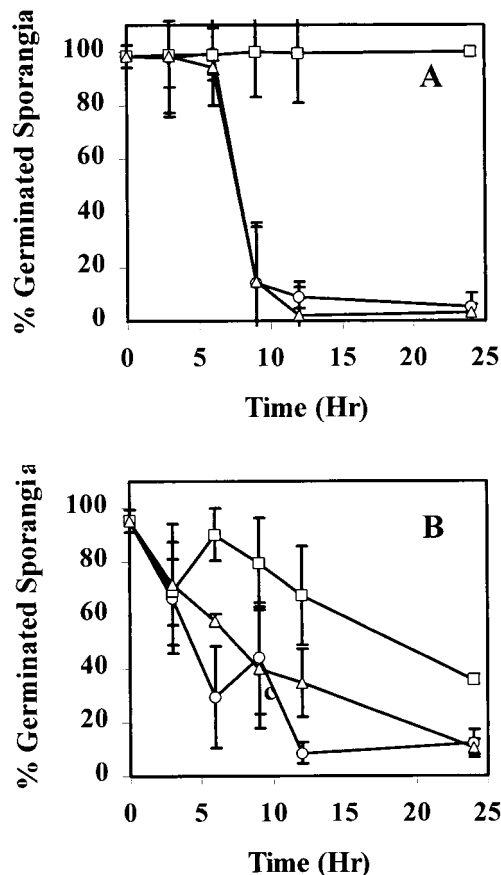


FIG. 2. Reduction in germination-stimulating activity of linoleic acid or cottonseed exudate due to growth of *E. cloacae* strains EcCT501R3 (○), Ec31 (□), and Ec31(pKV311) (△). Overnight cultures of *E. cloacae* were adjusted to a cell density of $10^9$ CFU/ml, Then, either 2 ml was placed in M9L medium (A) or 0.5 ml was placed in cottonseed exudate (15 mg/ml) (B). Cell aliquots of cultures at 3, 6, 9, 12, and 24 h were assessed for remaining germination-stimulating activity. The error bars represent the standard deviations. The error bars at some 24-h points are smaller than the marker.

the germination-stimulating activity of cottonseed exudate. *E. cloacae* strains EcCT-501R3, Ec31, and Ec31(pKV311) all grew similarly on cottonseed exudate (data not shown). However, Ec31 could not inactivate the germination-stimulating activity of cottonseed exudate as quickly and to the same level as that of EcCT-501R3 and Ec31(pKV311) (Fig. 2B). In one assay, EcCT-501R3 and Ec31(pKV311) reduced the germina-

TABLE 2. Linoleic acid levels in M9L after growth of *E. cloacae* strains

| *E. cloacae* strain | Remaining linoleic acid levels (mg/ml)[a] at: | | |
|---|---|---|---|
| | 0 h | 3 h | 12 h |
| EcCt-501R3 | 0.35 | 0.14* | 0.00* |
| Ec31 | 0.35 | 0.35 | 0.32 |
| Ec31(pKV311) | 0.35 | 0.03* | 0.00* |
| EcL1 | 0.35 | 0.35 | 0.38 |
| EcL1 (pKVL16) | 0.35 | 0.08* | 0.00* |

[a] Linoleic acid concentrations were determined by gas chromatography after periods of *E. cloacae* growth in M9 supplemented with 0.35 mg of sodium linoleate per ml. Numbers in each row followed by an asterisk differ significantly ($P = 0.05$) from the readings at 0 h according to *t* tests.

tion stimulating activity to 5 to 10% within 3 h, while Ec31 reduced the germination stimulating activity to only 35 to 40% after 24 h of growth. When linoleic acid was added to EcCT-501R3 treated exudate to ensure that the inactivation of cottonseed exudate germination-stimulating activity by EcCT-501R3 was not due to the production of sporangium germination inhibitors, the germination-stimulating activity was restored to 100% compared to 1% in response to the EcCT-501R3-treated exudate (data not shown).

**_E. cloacae_ EcCT-501R3 has a _fadL_ homolog necessary for linoleic acid metabolism by _E. cloacae_.** Because of the partial ability of Ec31 to inactivate cottonseed exudate germination-stimulating activity, we constructed an additional _E. cloacae_ mutant with a disrupted _fadL_ gene, which, in _E. coli_, encodes an outer membrane protein necessary for fatty acid uptake. Of approximately 1,500 cosmids screened, one clone, pKVL1, hybridized strongly to an _E. coli fadL_ probe. Sequencing revealed _fadL_ to be 1,329 bp, with a deduced product of 442 amino acids, and to have 78.0% identity with _E. coli fadL_. A Δ_fadL_::_nptII_ construct was marker exchanged into the chromosome of EcCT-501R3, producing mutant EcL1. Marker exchange was confirmed by the Km resistant (Km$^r$), Cm-sensitive (Cm$^s$) phenotype of EcL1, by Southern analysis, and by the failure of EcL1 to grow on linoleic acid as a sole carbon source (data not shown). EcL1 was complemented with pKVL16, which contained the _E. cloacae fadL_ open reading frame, and was restored in its ability to grow on linoleic acid as a sole carbon source (data not shown).

**_E. cloacae_ EcL1 cannot inactivate linoleic acid germination-stimulating activity and has a reduced ability to inactivate cottonseed exudate germination-stimulating activity.** EcL1 possessed stimulant inactivation phenotypes similar to those of Ec31 (Fig. 3). EcL1 M9L culture filtrates induced 80 to 90% sporangium germination even after 24 h of incubation, whereas EcCT-501R3 and EcL1(pKVL16) removed the majority of the linoleic acid germination-stimulating activity within 9 h (15 to 20% sporangium germination) (Fig. 3A). As with Ec31, EcL1 also failed to inactivate the germination-stimulating activity of other selected unsaturated long-chain fatty acids (data not shown). Gas chromatographic analysis of culture filtrates confirmed that EcL1 failed to remove linoleic acid from the growth medium (Table 2). EcL1 was also impaired in its inactivation of cottonseed exudate germination-stimulating activity (Fig. 3B). After 12 h of growth on cottonseed exudate, a germination-stimulating activity of 65% remained in EcL1 culture filtrates, whereas a germination-stimulating activity of 10% remained in culture filtrates of EcCT-501R3 and EcL1(pKVL16).

**Neither Ec31 nor EcL1 can protect cotton seeds from _P. ultimum_ seed infections.** The effect of a _fadBA_ or a _fadL_ mutation on the biocontrol activity of _E. cloacae_ EcCT-510R3 against _P. ultimum_-incited seed rot was tested in cotton seedling bioassays using a _Pythium_-infested soil. Both Ec31 and EcL1 failed to suppress _Pythium_ seed rot (Table 3, Fig. 4). EcL1 or Ec31 populations of 6 × 10$^9$ per soil column gave rise to 13.3% or 6.7% seedling stands, respectively, and the seedling quality was poor (ratings of 0.8 and 0.4, respectively). Both pKV311 and pKVL16 restored the biocontrol ability of Ec31 or EcL1, respectively, giving rise to ≥80% seedling stands (Table 3, Fig. 4). Seedling stands arising from seeds treated with complemented mutant strains did not differ significantly from those treated with the wild-type strain. Moreover, seedling quality from seeds treated with EcL1(pKVL16) or Ec31(pKV311) did not differ significantly from seedling quality arising from seeds treated with EcCT-501R3.
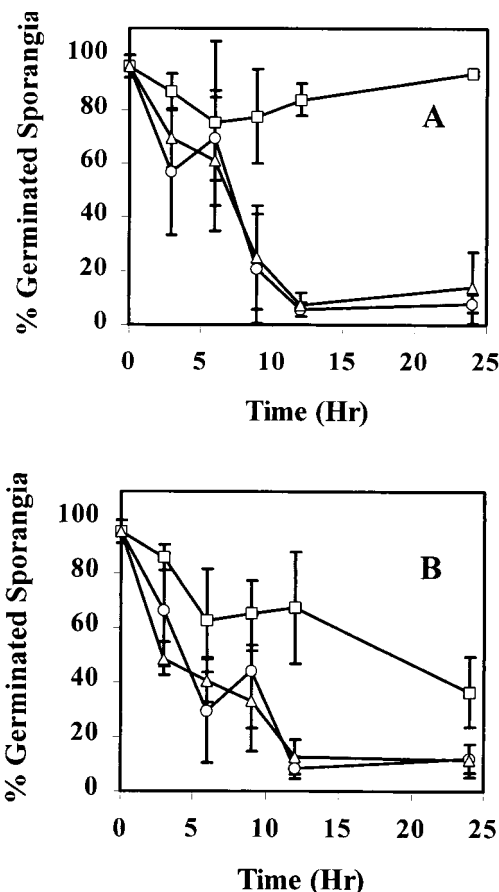


FIG. 3. Reduction in germination-stimulating activity of linoleic acid or cottonseed exudate due to growth of _E. cloacae_ strains EcCT501R3 (○), EcL1 (□), and EcL1(pKVL16) (△). Overnight cultures of _E. cloacae_ were adjusted to a cell density of 10$^9$ cfu/ml. Then, either 2 ml was placed in M9L medium (A) or 0.5 ml was placed in cottonseed exudate (15 mg/ml) (B) Cell aliquots of cultures at 3, 6, 9, 12, and 24 h were assessed for remaining germination-stimulating activity. The error bars represent the standard deviations. The error bars at some 24-h points are smaller than the marker.

## DISCUSSION

Our results provide strong evidence for fatty acid competition in the biological control of _Pythium_ damping-off by _E. cloacae_. The dependency of _P. ultimum_ developmental processes on fatty acids and other lipids (19, 25, 31, 56), coupled with the ability of _E. cloacae_ to rapidly metabolize fatty acids, provides ideal conditions for competitive interactions between _E. cloacae_ and _P. ultimum_. The strength of our conclusions comes from evidence from our current and past studies in which we have identified a common fatty acid resource and established its importance to the behavior of _P. ultimum_ (45, 53). Furthermore, we have demonstrated here a mechanism by which _E. cloacae_ can limit the availability of fatty acids to _P. ultimum_, thus establishing competition in favor of _E. cloacae_.

Conclusions have been made, based on other biological control studies with root-infecting pathogens, that microbial competition may be involved in the suppression of diseases (42). However, insufficient information is currently available to determine the limiting resources shared between the pathogens and introduced microbial inoculants. Studies with microbial siderophores have provided the only indirect evidence for iron

TABLE 3. Suppression of *Pythium* seed rot of cotton by wild-type and fatty acid mutant strains of *E. cloacae*

| *E. cloacae* strain | Seedling stand[a] (%) | Seedling quality[b] |
|---|---|---|
| Noninoculated Control | 86.7 | 2.5 |
| Nontreated (*Pythium* only) | 6.7* | 0.8* |
| EcCT-501R3 | 93.3 | 3.0 |
| Ec31 | 6.7* | 0.4* |
| Ec31(pKV311) | 86.7 | 2.6 |
| EcL1 | 13.3* | 0.8* |
| EcL1(pKVL16) | 80.0 | 2.4 |

[a] Seedling stands determined 7 days after sowing. Percent stands are based on five individual seedlings per treatment. The results are the means of three replicate experiments. Values followed by an asterisk differ significantly from the noninoculated control according to simple *t*-tests.

[b] Disease severity rated as on a scale of 0 to 3, where 0 = no seedling emergence or emerged seedling but completely covered with mycelium; 1 = the emerged seedlings failed to develop and had visible mycelial growth; 2 = seedlings appeared healthy but had visible mycelial growth or seedlings were less vigorous than those in the noninoculated control cylinders; and 3 = seedlings were healthy and had no visible signs of mycelial growth. Values followed by an asterisk differ significantly from the noninoculated control according to the Mann-Whitney test.

competition in the biological control of some root-infecting pathogens (3, 29). However, the shared resources that may be potentially limiting to the interacting microbial populations have not been defined. Additionally, the iron requirements of the pathogens under study are not known and have only been inferred in a limited number of cases (8, 9, 27, 47). As a result, no solid evidence for iron competition exists, particularly from the perspective of the pathogen.

Our evidence for fatty acid competition between *E. cloacae* and *P. ultimum* comes not only from our understanding of the response of *P. ultimum* to fatty acids but from our characterization of fatty acid uptake and β-oxidation mutants (*fadB* and *fadL*) of *E. cloacae* EcCT-501R3. Based on our analysis of Ec31 and EcL1, the only differences between these strains and the wild type are dysfunctional *fadB* and *fadL* genes, respectively. Therefore, differences between the wild-type strain and the mutant strains in biological control efficacy, as well as reductions in germination-stimulating activity in the presence of seed exudates or linoleic acid, can be ascribed to these genes and their respective functions in β-oxidation.

The failure of Ec31 to grow on linoleic acid or reduce its germination-stimulating activity provides indirect evidence that the product of *fadB* is required for the β-oxidation of fatty acids. This β-oxidation impairment gave rise to several important phenotypes. First and most importantly, Ec31 failed to protect seeds from *Pythium* infections. Second, Ec31 failed to metabolize and thus inactivate the germination-stimulating activity of linoleic acid over a 24-h period. Third, Ec31 displayed a reduced ability to inactivate the germination-stimulating activity of cottonseed exudate compared to EcCT-501R3. Since the growth of Ec31 on seed exudate was similar to that of EcCT-501R3 over a 24-h period, this response cannot be explained simply by a general growth defect. Furthermore, since suppression of seed infections by *P. ultimum* must occur during the first 12 to 24 h after planting (15, 38, 40, 41), the inability of Ec31 to protect seeds from *Pythium* infection was due most likely to its reduced ability to remove fatty acids from the spermosphere during this time period.

Despite the dysfunctional *fadB* gene and the inability to reduce the germination-stimulating activity of linoleic acid and other unsaturated long-chain fatty acids, Ec31 retained some ability to inactivate the germination stimulating activity of cot-
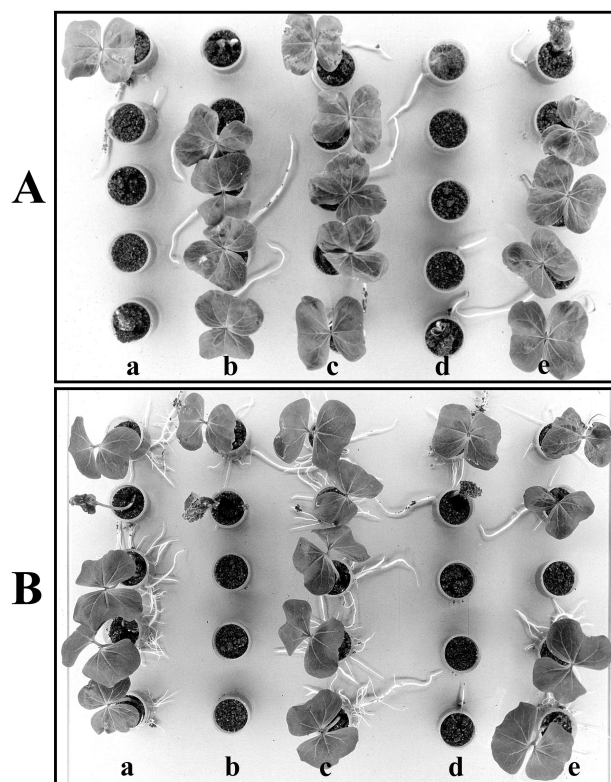


FIG. 4. Cottonseed bioassays illustrating the ability of *E. cloacae* strains to suppress *P. ultimum* seed rot. The bioassays were incubated at 24°C with a 16-h photoperiod and were photographed 7 days later. (A) Cotton seeds were placed in *P. ultimum*-infested soil and drenched with $KPO_4$ buffer (pH 7.0) (a), EcCT-501R3 (c) Ec31 (d), or Ec31(pKV311) (e). A noninoculated control is shown in column b. (B) Cottonseeds were placed in *P. ultimum*-infested soil and drenched with $KPO_4$ buffer (pH7.0) (b), EcCT-501R3 (c) EcL1 (d), and EcL1(pKVL16) (e). A noninoculated control is shown in column a.

tonseed exudate. There are a number of possible explanations for this. First, although fatty acids comprise most of the germination-stimulating activity in cottonseed exudate, there are additional lipid exudate fractions that contain low levels of germination-stimulating activity (45). It is therefore possible that either Ec31 can still metabolize these weakly stimulatory molecules and thus slightly reduce cottonseed exudate germination-stimulating activity. Another possibility is that Ec31 can still take up and partially degrade fatty acids, albeit at a much slower rate than EcCT-501R3. Such a process has been described in β-oxidation mutants of *E. coli* (26).

Because Ec31 retained some capacity to inactivate a significant amount of cottonseed exudate germination-stimulating activity, we constructed a *fadL* mutant of *E. cloacae*, EcL1, since *fadL* is absolutely required for long-chain fatty acid uptake in *E. coli*. EcL1 shared similar phenotypes with Ec31. Like Ec31, EcL1 was reduced, but not completely impaired, in its ability to inactivate cottonseed exudate germination-stimulating activity. Its growth rate on cottonseed exudate was similar to that of EcCT-501R3 and Ec31 over a 24-h period. This indicates that compounds other than fatty acids in cottonseed exudate may also act as low-level stimulants for *Pythium* sporangium germination and that the ability of EcL1 and Ec31 to partially reduce the germination-stimulating activity of cottonseed exudate but not that of linoleic acid is due to the metabolism of molecules other than fatty acids with slight germination-stimulating activity. However, the inability to completely

reduce the germination-stimulating activity of cottonseed exudate was sufficient to impair the capacity of both *E. cloacae* EcL1 and Ec31 to suppress *Pythium* seed infection.

Biological control of fungal pathogens by seed- and root-associated bacteria is undoubtedly a complex process, requiring a number of traits for a bacterium to be successful in suppressing diseases. Despite the importance of fatty acid competition in the suppression of *Pythium* seed infections by *E. cloacae*, it is unlikely that this trait alone determines the success or failure of *E. cloacae* as a seed protectant. Some of the other traits may be related to (i) complex surface phenomena that place bacteria in positions to suppress *Pythium* infections (21, 38), (ii) to the rapid growth rate around seeds (13), (iii) to the ability to produce antifungal volatiles (22), and (iv) to the ability to compete with other spermosphere organisms. A better understanding of each mechanism in this and other complex biological control systems will provide important insights into the nature of microbial interactions in soil habitats and ways in which such interactions may be exploited for the development of ecologically based plant disease control strategies.

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