Attachment of *Enterobacter cloacae* to Hyphae of *Pythium ultimum*: Possible Role in the Biological Control of Pythium Preemergence Damping-Off

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ABSTRACT

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Interactions between *Enterobacter cloacae* and *Pythium ultimum* were evaluated to determine the possible mechanisms by which *E. cloacae* protects plants against Pythium preemergence damping-off. In vitro, all strains of *E. cloacae* tested induced growth reductions of *P. ultimum* ranging from 33 to 54%. Antibiotics, toxins, or cell-wall-degrading enzymes were not detected in culture filtrates. In culture media amended with D-glucose, D-galactose, sucrose, *N*-acetyl-D-glucosamine, or β-methyl-D-glucoside, *E. cloacae* caused little or no reductions in growth of *P. ultimum*. However, in the absence of sugar or in the presence of D-raffinose, L-sorbose, 3-*O*-methyl-D-glucose, or α-methyl-D-glucoside, reductions in mycelial dry weight of *P. ultimum* due to *E. cloacae* ranged from 16 to 41%. Growth inhibition of *P. ultimum* was associated with the binding of *E. cloacae* to fungal hyphae as determined from scanning electron microscopy and from agglutination of cell wall fragments of *P. ultimum* by *E. cloacae*. Sugars that effectively blocked binding and cell wall agglutination also reduced the ability of *E. cloacae* to inhibit growth of *P. ultimum*. When *E. cloacae* was evaluated as a biological control agent on a variety of plant species, those whose seeds exuded high levels of carbohydrates were not protected by *E. cloacae* and the rate of seed colonization by *Pythium* spp. was the same for bacterized and nonbacterized seeds. *E. cloacae* was effective as a biological control agent only when coated onto seeds of species that exuded low levels of carbohydrates during germination. Treatment of cucumber seeds (low sugar exudation) with *E. cloacae*, in combination with sugars that interfere with bacterial binding to hyphae of *P. ultimum* in-vitro, effectively eliminated biological control activity in soil without directly stimulating fungal colonization of cucumber seeds by *Pythium* spp. Seed colonization by *Pythium* spp. was not increased compared with bacterized seeds nor was biological control activity eliminated when sugars that do not interfere with the binding of *E. cloacae* to hyphae of *P. ultimum* were incorporated into seed treatments. It was concluded that the ability of *E. cloacae* to function as a biological control agent on seed surfaces was directly related to its ability to bind to hyphae of seed colonizing *Pythium* spp. and to inhibit further hyphal development and infection at the seed surface.

Additional key words: agglutinin, lectinlike inhibitor, seed treatment.

Enterobacter cloacae (Jordan) Hormaeche and Edwards is an effective biological control agent against seed and seedling rots incited in cucumbers, peas, and beets by *Pythium*(14). It is one of the most abundant bacteria found in the spermosphere and rhizosphere of a variety of plant species (19,21,39,44) and may be a promising biological control agent against soilborne diseases of these and other agriculturally important plants.

The mechanism(s) by which *E. cloacae* protects seeds and seedlings is unknown, although events important in biological control mechanisms have been elucidated with other bacterial antagonists of *Pythium* spp. Howell and Stipanovic (15) demonstrated that the antibiotic, pyoluteorin, produced by a cotton rhizosphere isolate of *Pseudomonas fluorescens* (Trevisan) Migula was responsible for the in vitro inhibition of the growth of *Pythium ultimum* Trow. Treating cotton seeds with either the bacterium or the antibiotic effectively controlled Pythium damping-off. In vitro mycelial lysis of *Pythium debaryanum* Hesse, mediated by β-1,3-glucanase and protease enzymes produced by an *Arthrobacter* species, was shown to be responsible, at least in part, for the control of Pythium damping-off of tomato (29). In addition, mechanisms involving hyperparasitic events between the biological control agent, *Rhizobium japonicum* Kirchner, and *P. ultimum* have also been observed (45,46).

The purpose of this study was to evaluate specific interactions between *E. cloacae* and *P. ultimum* that influence the growth of *P. ultimum* and its colonization of seeds to determine which factors may mediate biological control of Pythium preemergence damping-off by *E. cloacae*.

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MATERIALS AND METHODS

Culture media and maintenance of organisms. Strains NRRL B-14095 and NRRL B-14096 of *E. cloacae* used in this study were isolated from cucumber (*Cucumis sativus* L.) seeds and have been described previously (14). Strains 0295, 0296, and 0297 of *E. cloacae* were donated by S. V. Beer, Cornell University. All cultures were maintained on yeast dextrose calcium carbonate agar slants containing (per liter): yeast extract (Difco Laboratories, Detroit, MI), 10 g; dextrose, 15 g; $CaCO_3$ (powdered), 20 g; and agar, 15 g.

Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) (50 ml/250-ml flask) was used to grow inoculum of *E. cloacae* in all in-vitro experiments and for treating seeds. In all experiments, cells were removed from the trypticase soy broth by centrifugation (10,000 *g* for 10 min at 4 C) after 24 hr of incubation (30 C). Before use, cells were washed by resuspending them in a saline solution (0.85% NaCl; w/v) and removed by centrifugation. In experiments with heat-killed cells, cultures of *E. cloacae* were autoclaved at 121 C for 15 min prior to centrifugation.

Isolates P4 (36) and PHP4 (obtained from grain-cropped soil [14]) of *Pythium ultimum* were used throughout this work. Both isolates of *P. ultimum* were maintained on Difco cornmeal agar (CMA). Sporangia were obtained from 2- to 3-wk-old cultures grown (25 C) on a dilute bean extract medium. This medium was prepared by autoclaving 10 g of frozen snapbeans in 1 L of water for 15 min. The beans were removed; the filtrate was dispensed into flasks (50 ml/500-ml flask), re-autoclaved, and seeded with a CMA culture of *P. ultimum*.

In most experiments with paired cultures of *P. ultimum* and *E. cloacae*, a sugar-mineral salts medium (SMS) was used which contained (per liter): K_2HPO_4 and KH_2PO_4 (autoclaved separately), 0.9 g each; KCl, 0.2 g; NH_4NO_3, 1.0 g; $CaCl_2$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4$, 2.0 mg; $MnSO_4$, 2.0 mg; $ZnSO_4$, 2.0 mg; thiamine HCl (autoclaved separately), 0.1 mg; a selected sugar (autoclaved separately), 1.0 g; and agar (optional), 20 g. In some experiments, SMS was modified by replacing NH_4NO_3 with either Difco casamino acids (5 g/L), a selected individual amino acid (0.4 g N/L), or soy lecithin (1 mg/ml). Soy lecithin was used because it supported the growth of *P. ultimum* but would not support the growth of *E. cloacae* (NRRL B-14095). Media were adjusted to pH 6.8–6.9 by adding 0.1 N HCl or 0.1 N NaOH. Cultures of *P. ultimum* used for scanning electron microscopy were grown on either 2% water agar (WA) or on WA containing D-glucose or sucrose (1 g/L).

Fungal growth inhibition. Single 2-mm-diameter agar disks from cultures of *P. ultimum* were placed in 10 ml of modified SMS liquid medium containing casamino acids and incubated at 25 C in 9-cm-diameter petri plates. After 48 hr, 0.1-ml aliquots of a washed suspension (10^8 cells per milliliter) of *E. cloacae* was added to cultures of *P. ultimum*. These paired cultures were incubated at 25 C and, after 48 or 72 hr, mycelial dry weight was determined by filtration through preweighed Whatman #1 filter papers, drying overnight at 60 C, and weighing.

Inhibitory activity of culture filtrates. Culture filtrates were assayed for the presence of inhibitory substances by filtering 48-hr paired cultures through Whatman #1 filter paper to remove nonsoluble constituents (e.g., hyphae, cell walls, or particulate soy lecithin). Filtrates were then sterilized by passing them through a 0.2-μm (pore size) filter (Nuclepore Corp., 7035 Commerce Circle, Pleasanton, CA) and freeze dried. Freeze-dried residues were redissolved in 1 ml of sterile distilled water and filter paper disks (12.7 mm diameter) which were saturated with the concentrated filtrate (0.1 ml/disk) were then placed around the periphery of a petri plate containing potato-dextrose agar (Difco Laboratories). An agar disk from a culture of *P. ultimum* was placed in the center of a petri plate and incubated at 25 C. After 3–4 days, cultures were examined for the presence of inhibition zones surrounding the filter paper disks.

Enzyme assays. The enzymes β-1,3-glucanase EC 3.2.1.39 and cellulase EC 3.2.1.4 were assayed according to Elad et al (10) from freeze-dried culture filtrates. Protease EC 3.2.1.4 activity was determined by dissolving freeze-dried culture filtrates in 2 ml of 0.05 M phosphate buffer (pH 8) and 1 ml of 0.1 M $MgCl_2$. Five milligrams of Azocoll (Sigma Chemical Co., St. Louis, MO) was added as a substrate and the reaction mixture was incubated on a rotary shaker (200 rpm) at 37 C for 1 hr when the reaction was stopped by adding 1 ml of 40% trichloroacetic acid. The mixture was kept at 4 C for 30 min to allow precipitation of insoluble constituents. Absorbance of the supernatant was then measured at 440 nm with a Gilford model 250 spectrophotometer. One protease unit (based on trypsin EC 3.4.21.4 as the standard) was defined as the activity that caused an absorbance change of 0.04 during 1 hr under these conditions.

Protein content of filtrates (based on bovine serum albumin as the standard) was determined by the method of Bradford (5) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Preparation of fungal cell walls. Isolates of *P. ultimum* (P4 or PHP4) were grown in petri plates containing 10 ml of a modified SMS liquid medium with D-glucose and casamino acids as the C and N source, respectively. After 5 days at 25 C, hyphal mats were removed from the medium, rinsed thoroughly with sterile distilled water, and comminuted for 2 min in an Omni Mixer (Sorvall Instruments, Newtown, CT). Approximately 50 ml of this suspension was placed in each of several plastic bottles and frozen. Hyphae were further disrupted by placing thawed bottles in a sonicator (Model W-370, Heat Systems Ultrasonics, Plainview, NY) equipped with a cup horn (Model 431 A) tuned to a frequency of 20 kHz. Samples were sonicated at 150 W for 10 min and then centrifuged (400 *g* for 1 min) to sediment most of the hyphal fragments. The supernatant was discarded and pellets were resuspended in sterile distilled water, sonicated, and centrifuged as before. Most of the hyphal fragments were free of cytoplasm after the second sonication step. Samples were then incubated at 4 C for 5 min to allow any undisrupted cells to settle. Hyphal fragments present in the supernatant were washed with sterile distilled water by centrifugation (six times at 1,000 *g* for 5 min each). After this washing procedure, samples were largely free of cytoplasmic contaminants as observed under phase-contrast microscopy. Cell wall preparations were then freeze-dried and stored at −20 C until used.

Cell wall agglutination assay. Freeze-dried cell wall fragments were resuspended in 0.5 M phosphate buffer (pH 6.8) at a concentration of 0.5 mg/ml and a 50 μl aliquot of this suspension was placed in the well of a depression slide. Cell wall agglutination by *E. cloacae* (NRRL B-14095) was examined by adding an additional 50 μl of either sterile distilled water, a sugar solution (7–20 mM), a suspension of *E. cloacae* (10^{10} cells per milliliter; final concentration 2×10^7 cells per milliliter) suspended in sterile saline solution, or a suspension of *E. cloacae* (10^{10} cells per milliliter) in a 7–20 mM solution of a selected sugar.

Extracellular polysaccharide of *E. cloacae* was used in some experiments. It was collected by filtration of the supernatant from 24-hr cultures of *E. cloacae* through a 0.2-μm pore size filter. Soluble extracellular polysaccharide was precipitated from the filtrate (34), freeze-dried, and resuspended in sterile distilled water (5 mg/ml). A 50-μl aliquot of this solution was added to cell wall suspensions at a final concentration of 2.5 mg/ml. Observations were made for 1 hr for agglutination activity with an inverted phase-contrast microscope.

Soil. An Arkport fine sandy loam (16) naturally infested with *Pythium* spp. (primarily *P. ultimum* [36]) was used in most in vivo experiments and was maintained moist at 16 C in covered containers until use. In some experiments, this soil was sterilized by autoclaving for 1 hr on three consecutive days. Sterile soil was reinfested with sporangia of *P. ultimum* by adding one 50-ml flask culture (described above) per 500 g of dry soil. This provided approximately 1,000–1,500 cfu of *Pythium* per gram of dry soil as determined by plating on a selective medium (27). Soil moisture was adjusted to 14% (w/w) (−72 mbar) prior to use in all experiments.

Seed treatments and seedling assays. The following plant species were used: pea (*Pisum sativum* L. 'Venus'), cucumber (*Cucumis sativus* L. 'Slicemaster'), snapbean (*Phaseolus vulgaris* L. 'Bush Blue Lake 274'), lima bean (*Phaseolus lunatus* L. 'A-1477'), soybean (*Glycine max* (L.) Merr. 'Traverse'), corn (*Zea mays* L. 'Jubilee'), and rye (*Secale cereale* L. [cultivar unknown]). To apply *E. cloacae* to seeds, washed bacterial cells were resuspended in 3 ml of a 1.5% (w/v) solution of Methocel A4C Premium (Dow Chemical Co., Midland, MI) which was used as an adhesive. This suspension contained 10^8–10^{10} cfu/ml. In some experiments, bacteria were first resuspended in 10 ml of a sugar solution containing either 20 mM D-glucose, L-sorbose, 3-*O*-methyl-D-glucose, β-methyl-D-glucoside, or 10 mM sucrose or D-trehalose. After incubating for 15 min at 25 C, cells were removed by centrifugation and resuspended in 3 ml of Methocel containing the same concentration of the sugar in which cells were first resuspended.

Cucumber and rye seeds were treated at the rate of 1 ml of suspension per 25 seeds while all other seeds were treated at the rate of 2 ml per 25 seeds. In all experiments, final levels of *E. cloacae* recovered from treated seeds were between 10^6 and 10^7 cfu per seed. In most experiments, seeds were then planted as described previously (14). However, in experiments with sugar-treated seeds, seeds were placed in soil infested with *Pythium* spp. for 12 hr then transplanted in uninfested soil. To determine the length of time critical for the control of *P. ultimum* by *E. cloacae*, seeds were placed in soil free of *Pythium* spp., removed at 4-hr intervals, and

placed in soil infested with *Pythium* spp. Seedling stands in all experiments were evaluated 7 days after planting.

Seed colonization assays. The effects of *E. cloacae* and sugars on seed colonization by *Pythium* spp. were determined by placing 10 cucumber seeds in petri plates filled with soil infested with *Pythium* spp. Seeds were incubated in a germinator at 25 C. Seeds were removed at 2-hr intervals over a 24-hr period, rinsed thoroughly with distilled water to remove adhering soil particles, and placed on moistened blotters. After 2–3 days of incubation on blotters in a germinator (25 C), seeds were observed for the presence of fungal growth on and around them. Developing colonies were observed in pure culture to verify their identity as *Pythium* spp.

Collection and analysis of seed exudates. One hundred seeds, undamaged and with no seed coat cracks, from each plant species were surface-sterilized by soaking them in a 0.5% NaOCl solution for 30 min and rinsing them with sterile distilled water. Ten subsamples of 10 seeds each were placed in 25-ml flasks containing 10 ml of sterile distilled water and incubated on a reciprocating shaker in the dark at 25 C. After radicle emergence (16–24 hr) the liquid was removed and checked for bacterial contamination by plating 0.1 ml onto one-third-strength trypticase soy agar. Contaminated exudate samples were discarded while uncontaminated samples were passed through a 0.2-μm filter (Nuclepore Corp.), placed in preweighed plastic bottles, freeze-dried, and stored at −20 C until use. Carbohydrate content of exudates was determined according to the method of Morris (30) as modified by Loewus (22) with sucrose as the standard.

Preparation of specimens for scanning electron microscopy. Surface interactions between *E. cloacae* and hyphae of *P. ultimum* were observed in vitro by placing coverslips coated with WA around the periphery of a petri plate containing either unamended WA or WA amended with 1 g/L glucose or sucrose. Plates were seeded with *P. ultimum* (isolate PHP4) and incubated at 25 C. After 2 days, plates were flooded with *E. cloacae* (10^8 cells per milliliter) suspended in solutions of either sterile saline, D-glucose (20 mM), or sucrose (10 mM), and incubated for 24 hr. Nonadhering bacteria were then removed by rinsing plates with 50 ml of either distilled water or a solution of D-glucose or sucrose. Coverslips were removed and specimens fixed in 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7) for at least 3 hr. Specimens were washed four times within 30 min with 0.05 M phosphate buffer and postfixed in a 1% OsO_4 in phosphate buffer (pH 6.8) for 24 hr. Fixed specimens were rinsed four times with distilled water and dehydrated in a graded ethanol series. Critical point-dried specimens were coated with gold and palladium and viewed in an AMR 1000A scanning electron microscope.

Interactions between *E. cloacae* and *P. ultimum* on seed surfaces were examined by placing *E. cloacae*-treated cucumber seeds in autoclaved soil infested with *P. ultimum* (PHP4) at 25 C. Seeds were removed after 12 hr and fixed in 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) for at least 3 hr and treated further as described above.

All experiments were repeated at least once and each experiment contained five replications. Data were analyzed by using Student's *t* tests or Duncan's new multiple range test.

RESULTS

Biological control of preemergence damping-off and inhibition of growth of *P. ultimum* by *E. cloacae*. All strains of *E. cloacae* tested increased the emergence of cucumber seedlings 40–62% compared to the control when coated onto cucumber seeds that were planted in soil naturally infested with *Pythium* spp. In addition, all strains of *E. cloacae* reduced growth of *P. ultimum* (PHP4) 33–54% after 72 hr when paired in vitro in SMS containing soy lecithin.

Freeze-dried filtrates from paired cultures of *P. ultimum* (PHP4) and *E. cloacae* (NRRL B-14095) were analyzed for the presence of extracellular antibiotic or lytic substances and fungal cell wall-degrading enzymes. No inhibitory activity could be detected in any of the filtrates, even after they were concentrated 50-fold. Similarly, extracellular β-1,3-glucanase, cellulase, and protease enzymes were not detected in culture filtrates of *E. cloacae* paired with either purified cell walls of *P. ultimum* or the intact thallus of *P. ultimum*. Although no inhibitors were detected in routine assays with cell wall fragments as the substrate, agglutination of fragments was always observed upon the addition of cells of *E. cloacae*.

Attachment to, and agglutination of, *P. ultimum* cell walls by *E. cloacae*. Addition of live cells of *E. cloacae* (NRRL B-14095) to a suspension of cell wall fragments of *P. ultimum* (PHP4) resulted in an immediate and strong agglutination of wall fragments (Fig. 1B). Autoclaved cells of *E. cloacae* did not agglutinate cell wall fragments of *P. ultimum*. Similarly, a bacteria-free crude extracellular polysaccharide did not agglutinate cell wall fragments of *P. ultimum* when added at concentrations of 2.5 mg/ml. Suspending cells of *E. cloacae* in saline, L-sorbose, D-raffinose, 3-*O*-methyl-D-glucose, or β-methyl-D-glucoside solutions did not block agglutination (Table 1). However, when cells of *E. cloacae* were suspended in solutions of D-glucose, D-galactose, sucrose, β-methyl-D-glucoside, or *N*-acetyl-D-glucosamine and added to cell wall suspensions of *P. ultimum*, agglutination by *E. cloacae* did not

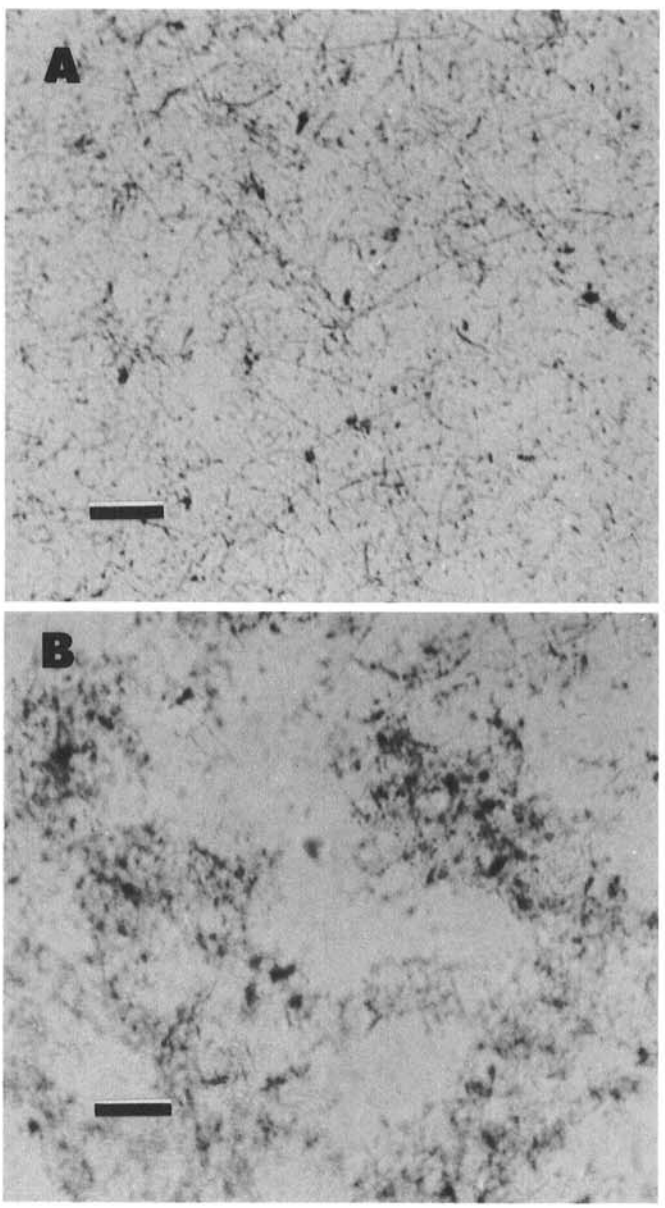


Fig. 1. Agglutination of cell wall fragments of *Pythium ultimum* (PHP4) by *Enterobacter cloacae* (NRRL B-14095). **A,** Cell wall preparation suspended in 0.05 M phosphate buffer (pH 6.8); **B,** cell wall preparation after addition of a cell suspension of *E. cloacae* (2×10^7 cells per milliliter). *E. cloacae* was suspended in 0.85% (w/v) NaCl solution. Bars ~ 180 μm.

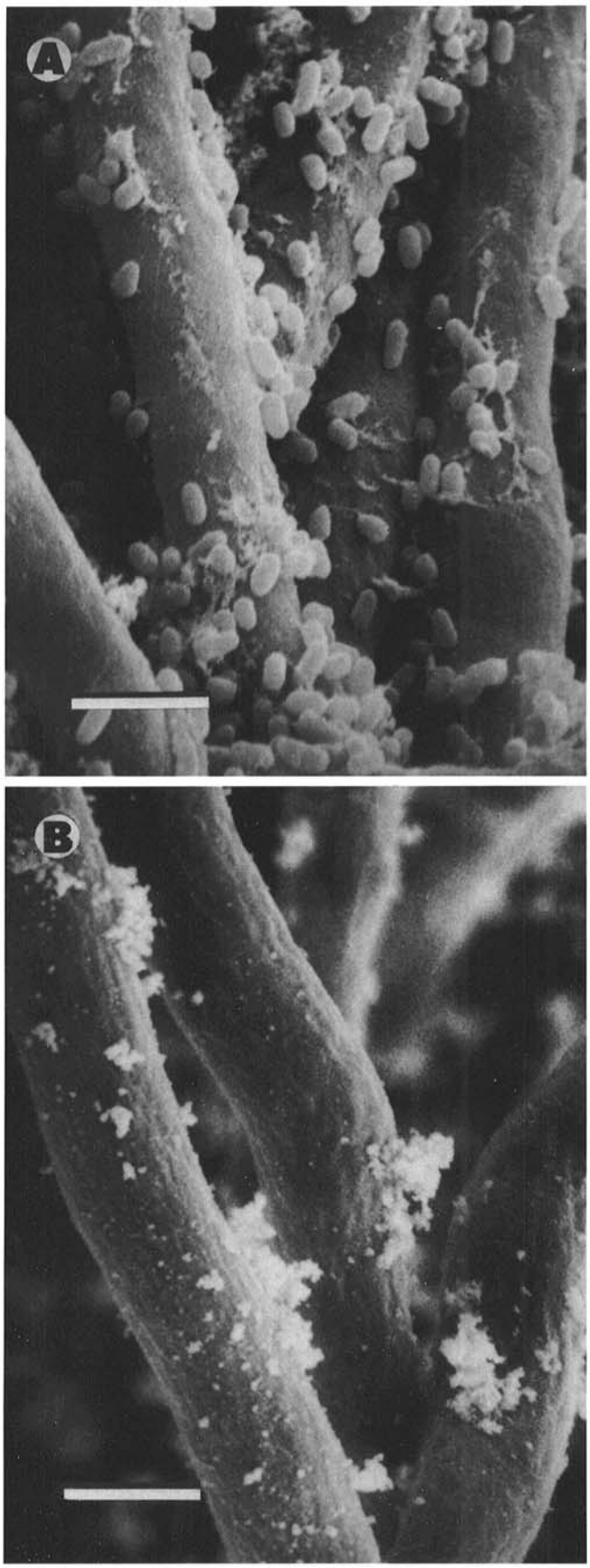


Fig. 2. Scanning electron micrographs of paired cultures of *Pythium ultimum* (PHP4) and *Enterobacter cloacae* (NRRL B-14095). **A,** No sugar added to paired cultures (after 24 hr, nonadhering bacteria were removed by rinsing paired cultures with distilled water); **B,** *P. ultimum* grown in the presence of 10 mM sucrose. After 24 hr, nonadhering bacteria were removed by rinsing paired cultures with a sucrose solution (10 mM). Bars ~ 5 μm.

occur. Only slight agglutination occurred in the presence of D-trehalose. Similar results were obtained with isolate P4 of *P. ultimum*.

In the absence of glucose or sucrose, cells of *E. cloacae* became firmly attached to living hyphae of *P. ultimum* (Fig. 2A). No localized sites of attachment were apparent from scanning electron microscopy observations and binding was more or less random over the hyphal surface. Polar attachment of bacteria was evident, although this was observed infrequently. In the presence of glucose or sucrose, *E. cloacae* was removed easily from hyphae by rinsing with glucose (20 mM) or sucrose (10 mM) solution (Fig. 2B).

Influence of sugars on the inhibition of growth of *P. ultimum* by *E. cloacae* (NRRL B-14095). Inhibition of growth of *P. ultimum* by *E. cloacae* was evaluated in media containing sugars which either prevented or had no effect on the agglutination of cell wall fragments of *P. ultimum* or attachment to hyphae. In all cases but one, *E. cloacae* did not significantly ($P = 0.05$) reduce growth of *P. ultimum* (PHP4) in media containing sugars which blocked the agglutination of cell walls of *P. ultimum* by *E. cloacae* (Table 1). For example, in media containing 20 mM D-glucose, 20 mM D-galactose, 10 mM sucrose, 18 mM *N*-acetyl-D-glucosamine, or 20 mM β-methyl-D-glucoside (sugars that block the agglutination of cell wall fragments of *P. ultimum*) mycelial dry weights of *P. ultimum* did not differ ($P = 0.05$) from axenic cultures of the fungus after 48 hr. When cultures were paired in the presence of 7 mM D-raffinose, 20 mM L-sorbose, or 20 mM β-methyl-D-glucoside (sugars that do not block the agglutination reaction), *E. cloacae* reduced growth of *P. ultimum* by 37, 41, and 20%, respectively. Pairing cultures of *P. ultimum* with *E. cloacae* in the presence of 10 mM D-trehalose did not result in a significant ($P = 0.05$) reduction in growth of *P. ultimum* even though slight agglutination of cell wall fragments by *E. cloacae* was observed.

Protective effects of *E. cloacae* (NRRL B-14095) against Pythium preemergence damping-off of various plant species. Protection against Pythium preemergence damping-off as a result of treating seeds with *E. cloacae* (NRRL B-14095) varied greatly depending upon the plant species even though the levels of *E. cloacae* on each plant species were similar (10^6–10^7 cfu per seed). For example, percent increases in emergence of snapbeans, lima beans, corn, peas, and soybeans due to *E. cloacae* were only 0, 4, 6, 13, and 14%, respectively, and were not significantly greater ($P = 0.05$) than from untreated seeds, while increases in rye and cucumber seedling emergence due to *E. cloacae* were 35 and 64%, respectively.

Seeds of the different plant species were analyzed to determine the levels of carbohydrates present in exudates during the first 24 hr of germination. Corn and legume seeds, which had the highest amounts of total exudate, also exuded the highest levels of carbohydrates (expressed as sucrose equivalents). For example, 202 mg of sucrose equivalents per 100 corn seeds, which represented 48% of the total seed exudate, were detected after 24 hr, Lima bean, soybean, snapbean, and pea exuded 96, 96, 85, and 27 mg of sucrose equivalents per 100 seeds, respectively. These levels of carbohydrates comprised 20–25% of the total seed exudate. Rye and cucumber seeds produced low levels of total exudate as well as low levels of carbohydrates. Only 19 and 1 mg of sucrose equivalents per 100 seeds were detected from rye and cucumber seeds, respectively, which represented 38 and 11%, respectively, of their total exudates.

An inverse relationship was found between the level of carbohydrate exudation from various seeds and the ability of *E. cloacae* to protect them (Fig. 3). Seeds with low levels of carbohydrate exudates were protected more effectively from preemergence damping-off by *E. cloacae* than were those with higher levels of carbohydrate exudation. The level of protection decreased logarithmically with linear increases in carbohydrate exudation.

Influence of *E. cloacae* (NRRL B-14095) and sugars on seed colonization by *P. ultimum* (PHP4) and *Pythium* spp. and the control of preemergence damping-off. Addition of *E. cloacae* to cucumber seeds reduced the level of seed colonization by *Pythium* spp. during the first 20 hr of germination in soil infested with

Pythium spp. (Fig. 4). Untreated seeds were colonized rapidly by *Pythium* spp.; colonization of the seeds was 90–100% after 10–12 hr and it remained at that level through at least 24 hr. Observations of hyphae of *Pythium* spp. on the surface of untreated seeds after 12 hr indicated that these hyphae were essentially free of bacteria although massive amounts of particulate matter coated the hyphal surfaces (Fig. 5A). Few colonizing hyphae could be found on seeds treated with *E. cloacae* after 12 hr. However, on the few hyphae present, bacteria, presumed to be largely *E. cloacae*, were not removed easily by rinsing seeds with water (Fig. 5B). One hundred percent seed colonization had occurred on both *E. cloacae*-treated and untreated seeds after 24 hr.

Colonization of cucumber seeds treated with either D-glucose, sucrose (Fig. 5C), D-trehalose, 3-*O*-methyl-D-glucose, or β-methyl-D-glucoside by *Pythium* spp. did not differ from that of untreated seeds; seed colonization was 100% after 8–12 hr. Incorporation of sucrose into cucumber seed treatments along with *E. cloacae* negated the suppressive effect of *E. cloacae* on seed colonization by *Pythium* spp. (Fig. 4) and few or no bacteria were attached to seed-colonizing hyphae of *Pythium* spp. after seeds treated with *E. cloacae* and sucrose were rinsed with water.

Addition of sugars to cucumber seeds did not significantly ($P = 0.05$) alter the level of preemergence damping-off compared with that of untreated seeds (Table 2). Bacterization of seeds with *E. cloacae* at the rate of 10^6–10^7 cfu per seed resulted in significant ($P = 0.05$) increases in seedling emergence (Table 2). However, when *E. cloacae* was first suspended in either D-glucose, D-galactose, sucrose, or β-methyl-D-glucoside (sugars that block the binding of *E. cloacae* to *P. ultimum* hyphae in vitro) and these respective sugars were added to bacterized seeds, seedling emergence was significantly ($P = 0.05$) reduced compared to that from seeds treated only with washed cells of *E. cloacae* (no added sugar). Incorporation of β-methyl-D-glucoside in the coating on bacterized seeds reduced seedling emergence to levels that did not differ ($P = 0.05$) from untreated or Methocel-treated seeds. However, if cells of *E. cloacae* were first suspended in sugars that do not block the in-vitro attachment of *E. cloacae* to *P. ultimum*, then suspended in a Methocel suspension containing the same respective sugars (3-*O*-methyl-D-glucose, D-trehalose, or L-sorbose), levels of seedling

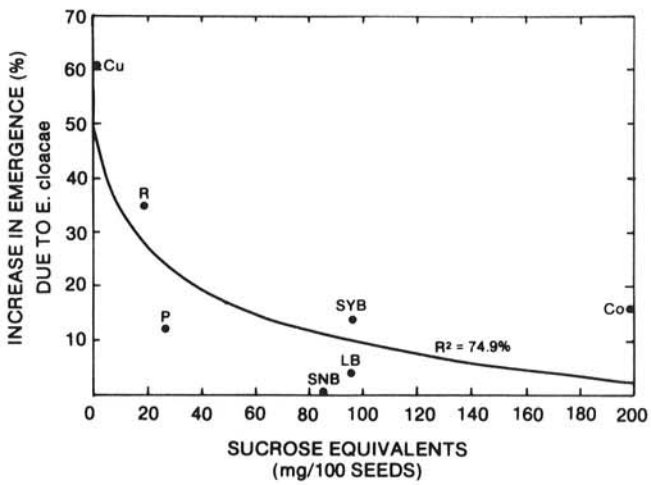


Fig. 3. Relationship between carbohydrate exudation from seeds and biological control activity of *Enterobacter cloacae* (NRRL B-14095). Legend: Cu = cucumber, R = rye, P = pea, SYB = soybean, LB = lima bean, SNB = snapbean, and Co = corn.

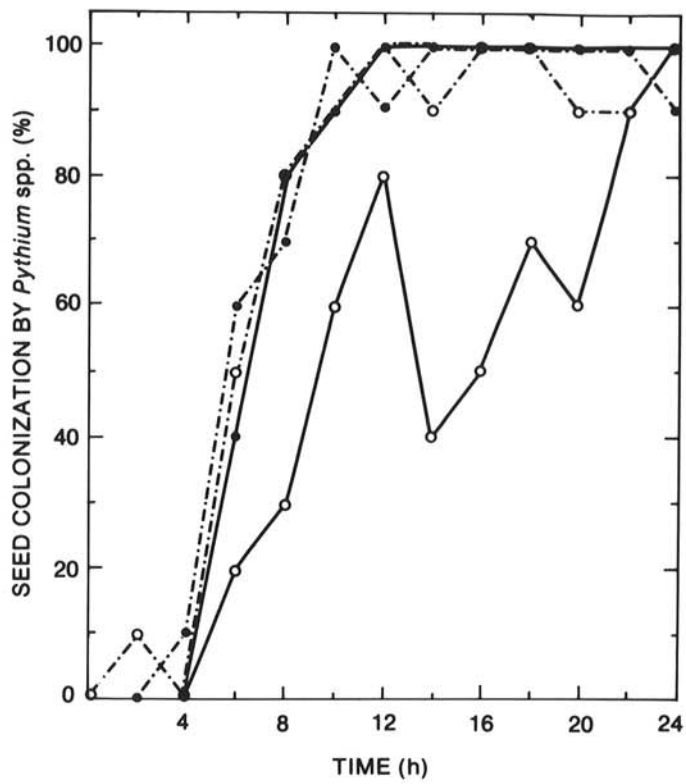
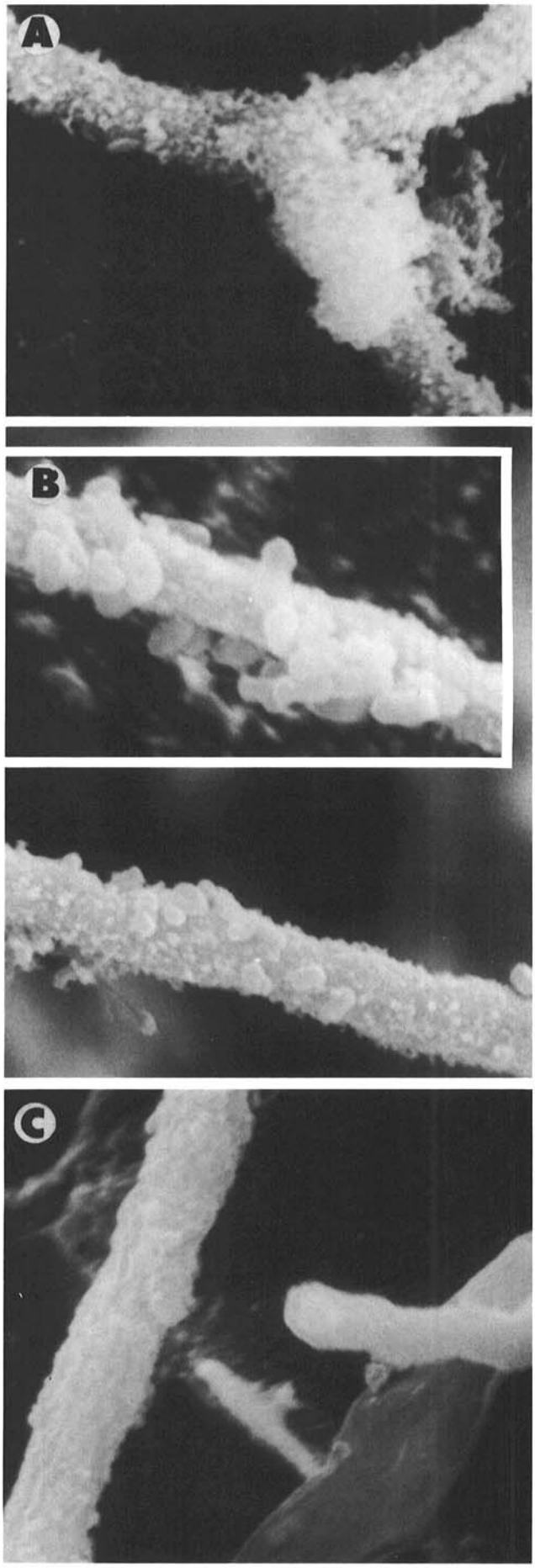


Fig. 4. Influence of seed treatments with sucrose and *Enterobacter cloacae* (NRRL B-14095) on the colonization of cucumber seeds by *Pythium* spp. during the first 24 hr of germination in soil. Legend: •——• = untreated seeds; •·····• = sucrose-treated (4 mg/ml) seeds; o——o = seeds treated (10^7 cfu per seed) with *E. cloacae*; o·····o = seeds treated with both sucrose (4 mg/ml) and *E. cloacae* (~10^7 cfu per seed). Soil populations of *Pythium* spp. were 1,415 cfu/g of soil.

TABLE 1. Influence of selected sugars on the ability of *Enterobacter cloacae* (NRRL B-14095) to agglutinate cell wall fragments and inhibit mycelial growth of *Pythum ultimum* (PHP4)

Sugar	Concentration (mM)	Cell wall agglutination[a]	Mycelial dry wt. (mg)[b]		Growth inhibition (% of control)
			No bacteria	*E. cloacae*	
Control (no sugar)	0	++	36.0	24.3*[c]	100.0
D-glucose	20	−	38.5	37.3	10.3
D-galactose	20	−	31.8	26.8	42.7
Sucrose	10	−	29.0	29.3	0.0
D-raffinose	7	++	32.0	21.0*	101.7
L-sorbose	20	++	31.8	18.7*	112.0
D-trehalose	10	+	21.7	20.4	11.1
3-O-methyl-D-glucose	20	++	33.5	23.9*	82.1
n-acetyl-D-glucosamine	18	−	35.8	32.9	24.8
α-methyl-D-glucoside	20	++	31.0	24.8*	53.0
β-methyl-D-glucoside	20	−	38.8	39.5	0.0

[a] Symbols: ++ = strong agglutination, + = slight agglutination, and − = no agglutination.
[b] Mycelial dry weights were recorded after 48 hr of incubation of the paired cultures.
[c] Asterisk (*) represents significant ($P = 0.05$) reductions in mycelial dry weights as compared with cultures to which no *E. cloacae* was added.



emergence did not differ ($P = 0.05$) from seeds treated only with washed cells of *E. cloacae* (no added sugar).

Transplant experiments. Incubation of untreated cucumber seeds for increasing periods of time in soil free of *Pythium* resulted in decreasing levels of preemergence damping-off when these were transplanted into soil infested with *Pythium* (Fig. 6). For example, seedling stands after 7 days in soil infested with *Pythium* following 0- and 12-hr preincubation periods in uninfested soil were 28 and 83%, respectively. Emergence of seedlings from seeds treated with *E. cloacae* and transplanted into soil infested with *Pythium* ranged from 90 to 100% after 7 days regardless of the preincubation time in soil free of *Pythium*.

DISCUSSION

Biological control of Pythium preemergence damping-off was common among the strains of *E. cloacae* that were evaluated. Previously established mechanisms of biological control mediated by antibiotic production or hyperparasitic events were not evident since no antifungal activity could be detected in concentrated filtrates from paired cultures of *E. cloacae* and *P. ultimum*. This is consistent with observations of Hadar et al (14) who found no inhibition of *P. ultimum* on solid media from bacterial mixtures containing *E. cloacae*. Likewise, extracellular cell wall-degrading enzymes suggestive of hyperparasitic interactions could not be detected in paired cultures of either *E. cloacae* and *P. ultimum* or *E. cloacae* and cell wall fragments of *P. ultimum*. Active destruction or invasion of hyphal surfaces was not evident from routine microscopical examination or from scanning electron microscopy of paired cultures. Therefore, there is no evidence for biological control to be the result of hyphal destruction from diffusable antibiotics, lytic substances, or direct parasitism.

A commonly observed feature of the *E. cloacae* - *P. ultimum* system in vitro was the ability of *E. cloacae* to attach to hyphae, agglutinate cell wall fragments of *P. ultimum*, and inhibit mycelial growth. The agglutination of cell wall fragments of *P. ultimum* occurred only in the absence of added sugars or in the presence of D-raffinose, L-sorbose, D-trehalose, 3-*O*-methyl-D-glucose, or α-methyl-D-glucoside. In the absence of sugar, cells of *E. cloacae* attached to hyphae and were not removed readily by rinsing with water. This is consistent with studies of phytoplanktonic bacteria where carbon limitation apparently promotes the attachment of bacteria to surfaces (6,24,25,49). However, in the presence of glucose or sucrose (sugars that block the agglutination of cell wall fragments by *E. cloacae*) bacteria did not attach to hyphae. This suggests that the binding of *E. cloacae* to hyphae of *P. ultimum* and agglutination of cell wall fragments may involve bacterial attachment to specific sugar residues associated with hyphae of *P. ultimum* and that prior exposure of *E. cloacae* to these or similar sugars may simply block available receptor sites.

Other studies have demonstrated the ability of many members of the Enterobacteriaceae, including *E. cloacae*, to attach to surfaces (40), including fungal hyphae (7), and to agglutinate a variety of materials containing complex carbohydrates (7,8,12). Many strains of *E. cloacae* produce cell-bound agglutinins, apparently associated with fimbriae, in which binding is blocked in the presence of D-mannose and α-methyl-D-mannoside (1). Other enteric bacteria produce up to three specific cell-bound agglutinins (8) as well as diffusable agglutinins (32). Apparently agglutinin-mediated attachment is critical in establishing pathogenic relationships in animal systems (8) as well as functioning in the adhesion of enteric bacteria to roots (13). In other systems,

Fig. 5. Influence of sucrose on the attachment of *Enterobacter cloacae* (NRRL B-14095) to seed-colonizing hyphae of *Pythium ultimum*. **A,** Hyphae from untreated cucumber seed; **B,** hyphae from cucumber seed treated with *E. cloacae* only ($\sim 10^7$ cfu per seed) (inset, ×10,000); and **C,** hyphae from seeds treated with *E. cloacae* and with 10 mM sucrose added. Seeds incubated for 12 hr in sterile soil infested with *P. ultimum*. Magnifications, ×5,200.

attachment of bacteria to fungal surfaces is important in establishing pathogenic relationships between the bacterium and the fungus (38,46), which results in detrimental effects on fungal growth and development.

Evidence from this study suggests there is a relationship between hyphal attachment, cell wall agglutination, and fungal growth inhibition. Significant growth inhibition of *P. ultimum* by *E. cloacae* occurred only in the absence of added sugars or in the presence of sugars (e.g., D-raffinose, L-sorbose, α-methyl-D-glucoside) which did not block the agglutination of cell wall fragments of *P. ultimum* and attachment to hyphae. Conversely, growth was unaffected in the presence of sugars (e.g., D-glucose, sucrose, β-methyl-D-glucoside) that blocked hyphal attachment by *E. cloacae* and the agglutination of cell wall fragments of *P. ultimum*. Therefore, hyphal attachment by *E. cloacae* appears to be a critical step in establishing antagonistic relationships with *Pythium* spp. Similar interactions have been observed in other systems (4) in which soil bacteria inhibit the growth of ectomycorrhizal fungi in media containing low but not high levels of glucose. However, the relationship between growth inhibition and attachment was not investigated.

The mechanism of adherence between *P. ultimum* and *E. cloacae* appears similar to the interaction between fungi and plant lectins (3,28,37). Mirelman et al (28) demonstrated that wheat germ agglutinin (WGA) binds specifically to hyphal tips of *Trichoderma viride* Pers. ex S. F. Gray and inhibits further hyphal extension, perhaps by preventing the incorporation of additional materials at the site of cell wall synthesis. Similarly, Barkai-Golan et al (3), using the soybean agglutinin and peanut agglutinin, demonstrated that lectin binding to hyphal tips and septa of *Penicillium* and *Aspergillus* spp. inhibits hyphal extension by preventing the incorporation of acetate, D-galactose, and *N*-acetyl-D-glucosamine into their chitinous cell walls. In addition, inhibition of spore germination as a result of lectin binding was also observed. Prior exposure of the lectins to their specific sugar inhibitors not only prevents binding but also prevents growth inhibition (3). Similarly, in the present study, prior exposure of *E. cloacae* to sugars that prevented binding to hyphae also prevented the inhibition of growth of *P. ultimum*.

Recent evidence suggests that specific adherence mechanisms may be important in bacterial-fungal (31,46), fungal-fungal (2,9), and fungal-nematode (33) interactions leading to the biological control of soilborne diseases. Antagonistic activity of *E. cloacae* was characterized in vitro by attachment-mediated inhibition of fungal growth, while antagonism in soil was expressed as reductions in preemergence damping-off resulting from the inhibition of seed colonization by *P. ultimum*. A direct relationship has been observed between the level of seed colonization by *Pythium* spp. and the level of preemergence damping-off (E. B. Nelson, G. T. Nash, and G. E. Harman, *unpublished*). Although reductions in the colonization of seeds treated with *E. cloacae* were not evident after 24 hr, reductions in seed colonization by *Pythium* spp. during the first 12 hr of seedling development appeared to be most critical for the expression of biological control. After 12 hr of germination in soil free of *Pythium*, cucumber seeds transplanted into soil infested with *Pythium* did not develop significant levels of preemergence damping-off. Similar results have been observed previously. Hadar et al (14) related the dramatic reductions in Pythium preemergence damping-off of cucumber after 8–24 hr of germination in-vitro to the increase in populations of native seed-surface strains of *E. cloacae* which multiply $\sim 10^2$ cfu per seed to $\sim 10^7$ cfu per seed during the first 8 hr of germination. Without bacterial development, seeds remain susceptible to preemergence damping-off (14). Biological control was effectively realized when recoverable seed populations of *E. cloacae* were between 10^6–10^7 cfu per seed during the initial 8–12 hr of germination in soil infested with *Pythium*. Apparently, events critical for attachment, as it affects biological control of Pythium preemergence damping-off, take place shortly after the seed is planted in soil (during the first 6–12 hr of germination) and events taking place after this period do not exert major influences on biological control or preemergence damping-off.

Just as sugars were the most evident factor correlated with the in vitro inhibition of growth of *P. ultimum*, the most apparent factor correlated with inhibition of seed colonization by *Pythium* and with biological control activity of *E. cloacae* was the level of carbohydrate present in seed exudates. The inability of *E. cloacae* to protect plants with seeds exhibiting high carbohydrate-exudation (e.g., legumes) from Pythium preemergence damping-off suggests that high exudate sugar levels in the spermosphere interfere with the binding of *E. cloacae* to hyphae of *Pythium* and subsequently reduce the level of suppression of *Pythium* by *E. cloacae* at the seed surface. This is supported by the observation that the extent of seed colonization by *Pythium* spp. on *E. cloacae*-treated pea and snapbean seeds, both of which exude high levels of carbohydrates, do not differ from levels of seed colonization by

TABLE 2. Influence of selected sugars on the ability of *Enterobacter cloacae* (NRRL B-14095) to protect cucumber seeds (cultivar Slicemaster) from Pythium preemergence damping-off[a]

Sugar	Concentration (mM)	Emergence (%)	
		No bacterium	*E. cloacae*[b]
Control (no sugar)	0	25 ef	98 a
3-O-methyl-D-glucose	20	5 f	90 ab
D-trehalose	10	5 f	80 ab
L-sorbose	20	10 f	78 ab
D-galactose	20	15 f	68 bc
D-glucose	20	10 f	65 bc
Sucrose	10	18 ef	55 cd
β-methyl-D-glucoside	20	18 ef	40 de

[a] Seeds placed in soil infested with *Pythium* (~500 cfu/g) for 16 hr then transplanted into uninfested soil. Seedling emergence evaluated 7 days after transplanting. Seedling emergence in soil free of *Pythium* was 100% for bacterized and nonbacterized seeds. Numbers followed by the same letter are not significantly ($P = 0.05$) different according to Duncan's new multiple range test.

[b] Cells of *E. cloacae* washed in the appropriate sugar solution and added to seeds at the rate of 10^6–10^7 cfu per seed.

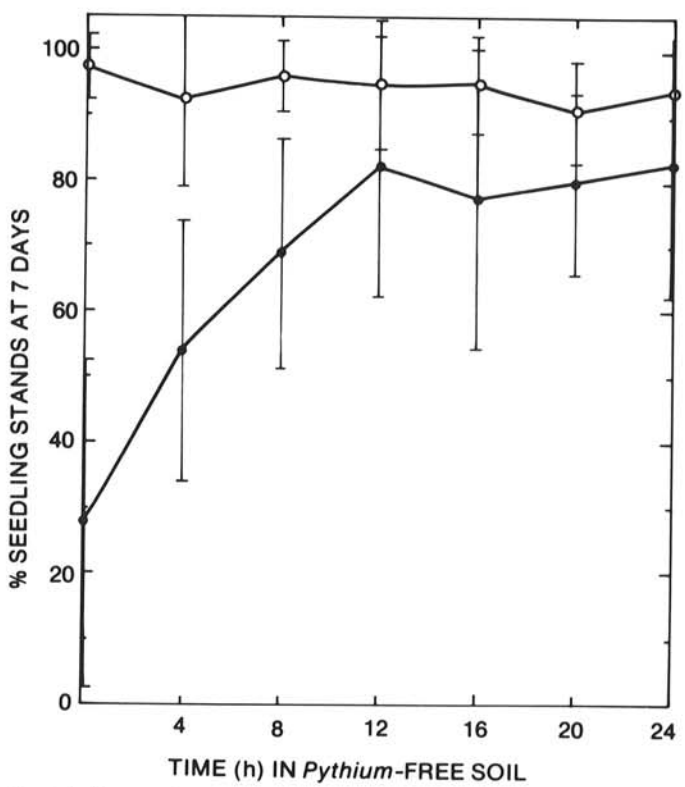


Fig. 6. Influence of germination time in the absence of *Pythium* spp. on the emergence of cucumber seedlings transplanted into soil infested with *Pythium*. Legend: •·····• = untreated seeds; o——o = seeds treated ($\sim 10^7$ cfu/g of soil) with *E. cloacae*.

Pythium or in amounts of preemergence damping-off relative to untreated seeds (E. B. Nelson, *unpublished*). The ability of exudate sugars to interfere with binding and growth inhibition of *Pythium* is further supported by the fact that seed exudate carbohydrates of a variety of plant species are composed primarily of the sugars sucrose, glucose, galactose, fructose, maltose, arabinose, and others (23,42,47,48). These sugars, particularly galactose, glucose, and sucrose, interfere with both the binding of *E. cloacae* to hyphae of *P. ultimum* and the inhibition of the growth of *P. ultimum*.

Artificially increasing the levels of specific sugars on the seed surface interfered with bacterial attachment to hyphae in the spermosphere. This not only influenced the extent of seed colonization by *Pythium* spp., but it also altered the ability of *E. cloacae* to function as a biocontrol agent. For example, coating cucumber seeds (low sugar exudation) with sugars that effectively blocked the attachment of *E. cloacae* to hyphae of *P. ultimum* in vitro (e.g., sucrose, β-methyl-D-glucoside) prevented bacterial attachment to hyphae of *P. ultimum* on seed surfaces, increased the level of seed colonization by *Pythium* spp., and inactivated *E. cloacae* as a biocontrol agent. On the other hand, treating cucumber seeds with sugars that did not interfere with binding and fungal growth inhibition (e.g., L-sorbose, 3-*O*-methyl-D-glucose), did not reduce the biocontrol activity of *E. cloacae*. Thus, conditions favoring attachment of *E. cloacae* to hyphae of *Pythium* and inhibition of hyphal development at the seed surface also favor the biological control of Pythium preemergence damping-off. Therefore, in vitro data concerning attachment of *E. cloacae* to *P. ultimum* and seed carbohydrate exudation could be used to reliably predict the behavior of *E. cloacae* as a biological control agent when it is applied as a seed treatment.

It is believed that sugars present in the spermosphere stimulate pathogens such as *Pythium* which in turn increase the levels of seed colonization, infection, and preemergence damping-off (11,17,18,23,26,35,41,43). Apparently, the addition of sugars to seeds eliminated biological control activity by blocking the attachment of *E. cloacae* to hyphae of *Pythium* at the seed surface instead of directly stimulating activity of *Pythium*, since levels of preemergence damping-off as well as the rate of seed colonization by *Pythium* spp. were not greater with sugar-treated (e.g., sucrose) seeds than with untreated seeds.

Knowledge of antagonist characters important in the biological control of soilborne diseases are known only in a very few instances (2,9,10,15). This lack of knowledge has probably contributed to our failure to reduce the variability in performance of biological control agents (20) and to successfully predict their behavior in soil. In the system involving *E. cloacae* and *P. ultimum*, we have described a binding character of *E. cloacae* that is necessary for this organism to function as a biological control agent. We feel that this is a first step in understanding what makes *E. cloacae*, and possibly other bacterial antagonists, effective biological control agents.

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