Production of Ammonia by *Enterobacter cloacae* and Its Possible Role in the Biological Control of Pythium Preemergence Damping-off by the Bacterium

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ABSTRACT

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Strains of *Enterobacter cloacae* reported to be biological control agents of seedling and postharvest diseases were found to symmetrically inhibit fungal growth when grown in dual cultures. Inhibition of fungal growth by *E. cloacae* in partitioned plates indicated that the inhibitor was volatile. Distillation and low-temperature trapping of released volatiles resulted in the capture of an antifungal compound that was identified as ammonia by mass spectroscopy. Assay of ammonia for activity against *Pythium ultimum* and *Rhizoctonia solani* showed that both were inhibited at low concentration and that the former was more sensitive than the latter. The addition of some sugars (D-galactose, D-glucose, sucrose, and β-methyl-D-glucoside) suppressed the production of ammonia by *E. cloacae* when added to the growth medium. Other sugars (3-*O*-methyl-D-glucose, D-trehalose, and L-sorbose) did not inhibit ammonia production. In a previous study, the sugars in the former group interfered with biocontrol activity, and the sugars in the latter group did not. These results indicate that ammonia production by *E. cloacae* may be the fungal growth-inhibiting part of the mechanism by which *E. cloacae* controls Pythium preemergence damping-off.

Additional keywords: seedling disease.

Enterobacter cloacae (Jordan) Hormaeche and Edwards, a nitrogen-fixing bacterium (6), has been shown to be an effective biological control agent of preemergence damping-off of pea, beet, cotton, and cucumber seedlings incited by *Pythium* species (3,10), of Fusarium wilt of cucumber (15), and of Rhizopus fruit rot of peach (17). An in-depth study of the mechanisms involved in the biological control by *E. cloacae* of seedling disease induced by *Pythium ultimum* Trow has shown a strong correlation between attachment of the bacterium to the hyphae of the fungus and inhibition of fungal growth and subsequent disease control (11). The presence of certain sugars in the environment prevented attachment of the bacterium to the fungus hyphae, inhibition of fungal growth, and disease control. None of the previous studies on the phenomenon of biocontrol with *E. cloacae* (3,10,11,15) have reported the presence of detectable antibiotic production or hyperparasitic activity by the bacterium, and the exact mechanism(s) of biological control remains somewhat obscure.

During a preliminary comparative study of *E. cloacae* strain ATCC 39979 (obtained from E. B. Nelson, Cornell University) and strain EcH-1 isolated from hyphae of *Pythium ultimum*, it was noted that, when inoculum of *Rhizoctonia solani* Kühn or *P. ultimum* was placed in dual culture with *E. cloacae* 72 hr after the bacterium, subsequent growth by either fungus was inhibited in a symmetrical fashion. Inhibition occurred without physical contact between the bacterium and the fungi.

The purpose of this study was to isolate and identify the inhibitory factor observed in the dual cultures and to determine its possible role in biological control.

MATERIALS AND METHODS

Isolation and maintenance of microorganisms. Strains ATCC 39979 and EcH-1 of *E. cloacae* were maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) for short-term experiments. Source material for both strains was maintained in 0.85% saline at 5 C. Strains J-1 of *R. solani* and Pu-1 of *P. ultimum* were maintained on potato-dextrose agar (PDA). Both fungi were isolated from infected cotton seedlings (*Gossypium hirsutum* L.). Strain EcH-1 was isolated from the mycelium of *P. ultimum* that had been exposed to the washings from dilution plates of cotton rhizosphere soil. After exposure for 30 min, the fungus mycelium was washed thoroughly six times with sterile water. The washed mycelium was macerated in a blender for 1 min in 50 ml of 0.85% sterile saline and diluted to 10^{-3}, and 0.1-ml aliquots were spread on TSA plates. After 3 days' incubation, colonies from bacterial cells that had remained attached to the hyphal fragments were isolated from the plates.

Characterization of the inhibitory factor. TSA plates were inoculated at two opposite locations around the periphery with *E. cloacae* and in the center with PDA plugs of *R. solani* or *P. ultimum* at intervals of 24, 48, 72, and 96 hr. After a 48-hr incubation at 25 C, plates were examined for evidence of fungal radial growth inhibition by the bacterium.

TSA plates were streak-inoculated with *E. cloacae*, and, after a 96-hr incubation, the cultures were minced and extracted with ethyl ether, ethyl acetate, butanol, methanol, or water. Extracts were concentrated to dryness in vacuo, and the residues were dissolved in small volumes of aqueous methanol (50:50, v/v). The concentrates were assayed for activity against *R. solani* or *P. ultimum* by placing 80-μl samples into wells cut into TSA medium at either end of the plate, and by inoculating in the center with the fungus. After 48 hr, the plates were examined for evidence of antifungal activity around the wells. Two sections of quad-partitioned plates containing TSA or TSA + 2% glucose were inoculated with *E. cloacae*. After a 96-hr incubation at 25 C, the remaining two sections were inoculated with *R. solani* and *P. ultimum*. After 24 and 48 hr, the plates were examined for evidence of fungal growth inhibition by the bacterium.

Quad-partitioned plates of TSA were inoculated in two sections with *E. cloacae*. After 4 days' incubation, the remaining sections were inoculated with PDA plugs of a *Fusarium* sp., *Verticillium dahliae* Kleb., *Thielaviopsis basicola* (Berk. & Br.) Ferr., and *Macrophomina phaseolina* (Tassi) Goid. After 48 hr, radial

growth of the fungi in dual culture with the bacterium was compared with fungal growth on uninoculated controls.

Isolation and identification of the fungal growth inhibitor. Fernbach flasks (2,800 ml) containing 200 ml of TSA or TSA + 2% glucose were spread-inoculated with a 1-ml suspension of *E. cloacae*. The flasks were sealed with # 12 stoppers perforated by two (5-mm-inside-diameter) glass tubes. One tube extended to within 1 cm of the agar surface, whereas the second tube extended only 1 cm below the rubber stopper. Each tube was plugged with sterile cotton. After 4 days' incubation at 25 C, cultures were placed in a 70 C water bath, and pressured air at the rate of 1.5 L/min was passed over the agar through the long inlet port and out of the short outlet port of the stopper. The air flow then was bubbled through a 5-ml water trap held near 0 C in a cryobath (Neslab CB-60, Neslab Instruments, Newington, NH). After 1 hr, the culture was replaced with a fresh one, and its contents were distilled into the same trap. The contents of the trap then were removed and stored in a sealed container in the freezer for further study.

Antifungal activities of the distillates from TSA cultures of *E. cloacae*, with and without glucose, were assayed by mixing 0.1, 0.2, 0.3, 0.4, or 0.5 ml of distillate with enough molten TSA to make 3 ml in small petri dishes. The plates were inoculated with PDA plugs of *R. solani* or *P. ultimum*, and, after 24 and 48 hr of incubation, radial growth was measured.

Samples of the distillates (2 μl) from cultures with and without glucose were subjected to mass spectral analyses with a VG-70-250 EHF integrated mass spectrometer. Samples were injected into an expansion inlet system, and the mass range from mass-to-charge ratio (m/z) 16 to 20 was monitored. Software peak matching then was used to determine the accurate mass of the suspected peak.

Assay of ammonia for antifungal activity. Varied amounts of ammonia (1–14 μl in 1-μl increments) in the form of ammonium hydroxide were added to 1-ml aliquots of sterile water in 3-cm-diameter petri dishes. Each was mixed with 2 ml of molten TSA, and the plates were inoculated with PDA plugs of *R. solani* or *P. ultimum*. After 24 and 48 hr of incubation at 25 C, radial growth of the fungi was measured and compared with untreated controls. In another test, varied quantities of ammonia (1–14 μl) in sterile water were placed in sections of quad-partitioned plates. The remaining sections were filled with TSA and inoculated and measured as above.

Effect of sugar and nitrogen-containing substrates on inhibitor production. The sugars D-glucose, D-galactose, L-sorbose, β-methyl-D-glucoside, and 3-*O*-methyl-D-glucose were added to TSA to a final concentration of 80 mM. Sucrose and D-trehalose were added to TSA to a final concentration of 40 mM. Aliquots (5 ml) of the sugar-containing agars were dispensed into two sections of quad-partitioned plates, whereas the other two sections were filled with TSA. Those sections with sugar were streaked with a suspension of *E. cloacae*, and the cultures were incubated at 25 C for 72 hr. Remaining sections then were inoculated with PDA plugs of *R. solani* or *P. ultimum* and incubated 24 hr, and fungal growth was compared with that on appropriate controls.

Sugar-mineral salts medium (11), modified by removal of NH_4NO_3, was used as a basal medium to assay the effect of different nitrogen sources on inhibitor production. Asparagine, glutamine, tryptophan, lysine, arginine, histidine, and tryptone were added to the basal medium (1% medium), autoclaved, and dispensed into two sections of quad-partitioned plates. The other two sections were filled with TSA. The nitrogen-containing sections of the plates were inoculated with loopfuls of *E. cloacae* cells and incubated 96 hr. Remaining sections of the plates then were inoculated with PDA plugs of *R. solani* or *P. ultimum*, and, after 24 and 48 hr of incubation, growth of the fungi was compared with controls.

All experiments were repeated one or more times with similar results, and each experiment contained three replications. Data were analyzed by using Student's t test.

RESULTS

Characterization of the inhibitory factor. Examination of dual cultures inoculated with PDA plugs of *R. solani* or *P. ultimum* 24, 48, 72, or 96 hr after the bacterium showed progressive symmetrical inhibition of the fungi, with complete inhibition occurring between 48 and 72 hr. No direct contact between the bacterium and the fungi was observed.

Assays of extracts of *E. cloacae*, made with solvents of varied polarity, were uniformly negative for antifungal activity. No detectable antibiotic activity was observed around the wells containing the extracts.

Examination of dual cultures of *E. cloacae* and *R. solani* or *P. ultimum* in quad-partitioned plates showed that fungal growth was inhibited by the bacterium, even though there was no direct contact between the medium supporting the respective cultures (Fig. 1). This indicated that the inhibitor was volatile. Agar medium in the uninoculated sections, when moved to another plate after exposure to the bacterium for 72 hr, still did not lose its inhibitory character when subsequently inoculated with *R. solani* or *P. ultimum*. This indicated that the inhibitory factor was absorbed and retained by the agar medium. *E. cloacae* grown on TSA medium containing 2% glucose did not inhibit the growth of either *R. solani* or *P. ultimum* when the bacterium and fungi were grown in dual-partitioned culture. Control cultures on TSA alone were completely inhibitory to both fungi.

Assay of the activity spectrum of the inhibitor by placing *E. cloacae* in dual culture with different fungi pathogenic to cotton showed that *E. cloacae* inhibited the growth of *V. dahliae*, *T. basicola*, and *M. phaseolina* completely, whereas the growth of a *Fusarium* sp. was inhibited by 50%.

Isolation and identification of the fungal growth inhibitor. Bioassay of the distillate from TSA and TSA + 2% glucose cultures of *E. cloacae* showed that there was antifungal activity in the former but not in the latter medium.

Exposure of the PDA plugs of *R. solani* or *P. ultimum* to varied concentrations of the active distillate showed that *P. ultimum* was more sensitive to the inhibitor than *R. solani*. Growth of *P. ultimum* was completely inhibited at the level of 0.3 ml of distillate per plate, whereas growth of *R. solani* was completely inhibited at 0.4 ml per plate.

Mass spectral analyses of the active TSA culture distillate and of the inactive TSA + 2% glucose culture distillate from m/z 14 to 150 showed that the only major difference in the spectra occurred at the exact mass of ammonia, m/z 17.0265 (calculation for NH_3: 17.0266) (Fig. 2). Areas of ammonia peaks were determined by digitizing the ion current spectra with a complot digitizer (Series 7000 digitizer, Houston Instruments, Austin, TX). Comparison of the m/z 17.0265 peak areas of the two spectra indicated that there was 24 times as much ammonia in the active distillate as there was in the inactive one.

Antifungal activity of ammonia. Examination of TSA plates containing 1–14 μl of ammonia per plate showed that *P. ultimum*

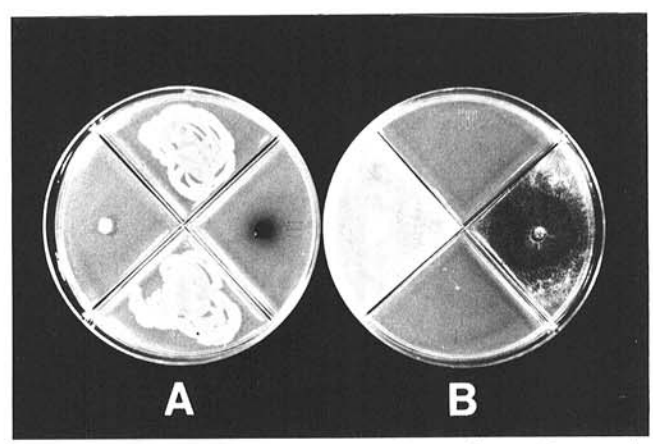

Fig. 1. Volatile inhibitory activity of *Enterobacter cloacae*. Comparative growth rates of *Pythium ultimum* (left sections) and *Rhizoctonia solani* (right sections) in (**A**) dual culture with *E. cloacae* or in (**B**) an uninoculated control.

was more sensitive to ammonia than *R. solani*. A concentration of 2 μl/plate was sufficient to completely inhibit *P. ultimum*, whereas 4 μl/plate was required to completely inhibit *R. solani* (Table 1). In those plates where the introduced ammonia was remote from the medium and the fungi, 8 μl inhibited the growth of *P. ultimum*, and 10 μl inhibited that of *R. solani*. Inhibition of fungal growth was characteristic of that observed in the presence of *E. cloacae*.

Effect of sugar and nitrogen-containing substrates on inhibitor production. Results of the sugar and nitrogen substrate tests for effect on inhibitor production are given in Table 2. D-glucose, D-galactose, sucrose, and β-methyl-D-glucoside all suppressed ammonia production by *E. cloacae* in vitro. L-sorbose and 3-*O*-methyl-D-glucose did not suppress, and D-trehalose only partially suppressed, ammonia production by *E. cloacae*.

Assay of different nitrogen-containing compounds as substrates for ammonia production by *E. cloacae* showed that asparagine, glutamine, and histidine were all good substrates for ammonia production, followed by tryptone. Arginine, lysine, and tryptophan were totally unsatisfactory as nitrogen sources for ammonia.

DISCUSSION

The results of our study indicate that the mechanisms involved in the biological control of Pythium preemergence damping-off by *E. cloacae* may be more complex than was previously believed. Other investigators have reported the absence of any detectable antibiotic production by *E. cloacae*. However, without knowledge of its volatility, an inhibitor could easily be missed. Our data show that *E. cloacae* does produce a strong antifungal compound inhibitory to many fungi and that this inhibitor is ammonia. Ammonia has been reported to be toxic to fungi in low concentration (8), to occur at fungistatic levels in soil (12), and to be generated by bacteria in plant tissue (1,7). Ammonia released from various nitrogenous compounds or in the form of anhydrous ammonia has been used as a fungicide (9,13,14,16) and to reduce disease severity (2).

Another interesting aspect of the biocontrol of *Pythium* spp. by *E. cloacae* is the discovery by Nelson et al (11) of the positive relationship between attachment of the bacterium to the hyphae of the fungus and subsequent fungal growth inhibition and disease control. These workers further found that the addition of certain sugars to the environment suppressed bacterial attachment, fungal growth inhibition, and disease control, whereas the addition of other sugars did not. Our data show that the sugars D-glucose, D-galactose, sucrose, and β-methyl-D-glucoside that suppressed disease control by *E. cloacae* on seed surfaces (11) are the same ones that suppress ammonia production by *E. cloacae* in vitro. The sugars 3-*O*-methyl-D-glucose, D-trehalose, and L-sorbose did not interfere with biocontrol when introduced on the seed with the bacterium (11), nor did they suppress ammonia production by *E. cloacae* in vitro in the present study. Therefore, the positive relationship between bacterial attachment to hyphae and disease control applies equally to ammonia production and control of the disease.

As pointed out by Nelson et al (11), *E. cloacae* is effective in disease control only on seeds that do not exude large amounts of sugar into the spermosphere during germination. These sugars interfere with bacterial attachment to fungi as well as ammonia production by *E. cloacae*. Ammonification of amino acids and acid amides in soils is well established (4), and their pattern of efficacy as sources of ammonia closely parallels that observed with *E. cloacae* in our tests. We believe it possible that, under conditions of low concentration of readily metabolized sugars in the spermosphere, *E. cloacae* deaminates amino acids exuded by the germinating seed to obtain a carbon source; thus, ammonia is produced as an antifungal byproduct of this process. Complementation of the hyphal attachment and ammonia production phenomena by *E. cloacae* would provide for on-site production of the inhibitor and, thus, would make it effective at a concentration lower than that required to saturate the soil environment. It also may nullify the problems associated with adsorption of ammonia by the soil colloids (5).

Although *E. cloacae* only inhibited the growth of *Fusarium* sp. by 50% in our study, it has been shown to effectively control Fusarium wilt of cucumber (15). This apparent activity difference is probably due to the form of the wilt pathogen in soil. Germination of chlamydospores and conidia is often suppressed at a much lower inhibitor concentration than that required to inhibit further growth of actively growing mycelium.

E. cloacae is not an effective biocontrol agent of seedling disease incited by *R. solani* (C. R. Howell, *unpublished*; E. B. Nelson, *personal communication*), even though the bacterium is inhibitory to the pathogen in vitro. This probably is due to the fact that *R. solani* is less sensitive to ammonia than *P. ultimum*, and infection by the pathogen usually occurs later in the development of the

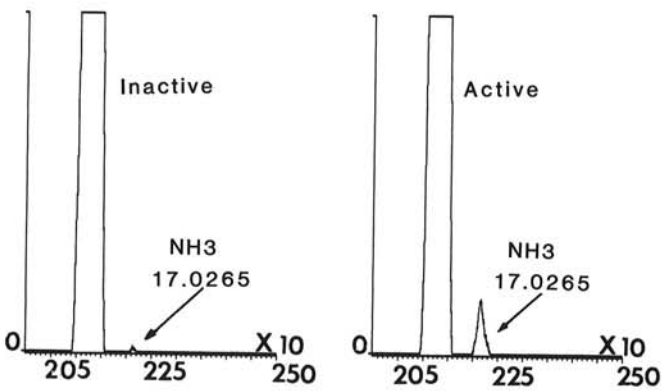

Fig. 2. Mass spectra of culture distillates of *Enterobacter cloacae* showing the relative ammonia (mass-to-charge ratio 17.0265) contents of inactive and active inhibitory fractions.

TABLE 1. Inhibitory activity of culture distillate of *Enterobacter cloacae* and ammonia on mycelial growth of *Pythium ultimum* and *Rhizoctonia solani*

Culture distillate[a] (ml)	Growth inhibition[b] (% of control)		Ammonia concentration (μl)	Growth inhibition (% of control)	
	Pu	Rs		Pu	Rs
0 control	0 ± 0[c]	0 ± 0	0 control	0 ± 0	0 ± 0
0.1	30 ± 2.5	0 ± 0	1	0 ± 0	0 ± 0
0.2	77 ± 3.5	25 ± 2.5	2	100 ± 0	54 ± 0
0.3	100 ± 0	65 ± 2.5	3	100 ± 0	68 ± 2.5
0.4	100 ± 0	100 ± 0	4	100 ± 0	100 ± 0

[a] Culture distillate was mixed with enough molten tryptic soy agar (TSA) to make 3 ml and poured into a 3-cm-diameter plate; NH_3 was added as NH_4OH to 1 ml of sterile H_2O and mixed with enough molten TSA to make 3 ml in a 3-cm-diameter plate.
[b] Pu = *Pythium ultimum*; Rs = *Rhizoctonia solani*.
[c] Values following the ± symbol are standard deviations from the means of three replicates.

TABLE 2. Effect of selected sugar and nitrogen substrates on fungal growth inhibitor production by *Enterobacter cloacae* (Ec)

Sugar	Growth inhibition[a] (% control)		Nitrogen	Growth inhibition (% control)	
	Pu	Rs		Pu	Rs
Control (no sugar)	100*[b]	100*	Control	0	0
L-Sorbose	99*	81*	Asparagine	100*	100*
3-*O*-Methyl-D-glucose	86*	76*	Glutamine	100*	100*
D-Trehalose	37*	28*	Histidine	100*	100*
D-Galactose	0	0	Tryptone	82*	68*
D-Glucose	0	0	Arginine	7	8
Sucrose	0	0	Lysine	7	8
β-Methyl-D-glucoside	0	0	Tryptophan	0	0

[a] Pu = *Pythium ultimum*; Rs = *Rhizoctonia solani*.
[b] Asterisk (*) represents significant ($P = 0.05$, Student's-*t* test) reduction in mycelial growth compared with cultures without *Enterobacter cloacae*.

seedling when the population of the bacterium may have diminished below a critical threshold.

In view of the fact that ammonia is highly toxic to *P. ultimum* at low concentration, that *E. cloacae* actively produces ammonia under conditions that might easily occur in the spermosphere, and that the pattern of sugar inhibition of ammonia production is consistent with that observed for suppression of biocontrol activity by *E. cloacae*, it appears that ammonia production may well be the inhibitory part of the mechanism by which *E. cloacae* controls Pythium preemergence damping-off. Confirmation of this hypothesis, through the removal and restoration of the capacity to produce ammonia in mutants of *E. cloacae*, is now under way.

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