Use of an Enzyme-Linked Immunosorbent Assay with Murine Ascitic Antibodies to Screen Microorganisms for Production of Cerato-ulmin, a Toxin of *Ceratocystis ulmi*

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ABSTRACT

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Polyclonal antibodies from hyperimmune ascitic fluid of mice immunized with cerato-ulmin (CU), a protein phytotoxin of *Ceratocystis ulmi*, have been utilized to develop a sensitive and specific assay to detect CU. As little as 20 μg (1.5 picomoles) of CU per assay well can be detected by this method. Employing extracts of plate cultures of a number of microbial species, including many isolated from elm tissue, as well as several other *Ceratocystis* spp., we noted that only extracts of *C. ulmi* contained antibody-reactive material and that 56 of 86 *C. ulmi* isolates screened for CU production were positive. The ELISA was compared with a turbidimetric method for detecting CU production in both extracts of plate cultures and cell-free culture filtrates of 10 *C. ulmi* isolates. Isolates classified as either producers or nonproducers of CU by the ELISA using extracts of plate cultures were found to group in the same categories when culture filtrates were assayed by either the ELISA or turbidimetry.

Two macromolecules isolated from cultures of *Ceratocystis ulmi* (Buism.) C. Moreau, the pathogen of Dutch elm disease, have been investigated for possible involvement in Dutch elm disease. The first, a peptidorhamnomannan, is released into culture filtrates during the growth of the organism (13,18,20,23) and is toxic at relatively low concentrations in bioassays with seedlings of *Ulmus americana* L. (23,32) but does not show biological specificity (23,32). Rabbit polyclonal (4,7,13,20) and monoclonal (3) antibodies against the peptidorhamnomannan have been reported. Although these have found application primarily in histological studies (2,4,7,20), none has been characterized with respect to cross-reactivity or specificity.

Cerato-ulmin (CU) (24), a small (M_r 13,500), hydrophobic, surface-active protein (15–17,21,22) produced by *C. ulmi*, is selectively toxic to elm (24). In vitro and in vivo studies have shown that CU plays a significant role in the development of Dutch elm disease (15,24,26,28,31).

Turbidimetry (30), used both to estimate the CU content of culture filtrates of *C. ulmi* and to screen isolates, has revealed a correlation between CU production and pathogenicity (6,14,15,26). Despite turbidimetry's sensitivity (30), the method is only semiquantitative and is not completely specific for CU. Rabbit antibodies to CU (7) and CU coupled to activated Dextran 70 (8) detected CU that had been administered to elm cuttings (7) or produced by *C. ulmi* in vivo (31). These antibodies were also not characterized as to specificity. A specific immunological probe for *C. ulmi* has a number of potential applications, including identifying the fungus, localizing the fungus and/or its toxin at the cellular or tissue level, providing a rapid and sensitive diagnostic tool, and monitoring gene expression by this fungus.

We have recently found that BALB/c mice injected with purified CU also elicit antibodies against this toxin and that ascitic fluid rich in anti-CU antibodies can be produced conveniently in the immunized mice that received sarcoma cells intraperitoneally (19). Using the enzyme-linked immunosorbent assay (ELISA), we have developed and applied a highly specific, sensitive, and accurate assay to measure CU produced by selected *C. ulmi* isolates grown either on plates or in liquid shake cultures and by numerous other fungal and bacterial isolates, many of which are associated with elm. The results of these investigations suggest that CU is probably unique to *C. ulmi*.

MATERIALS AND METHODS

Special reagents and chemicals. We purchased alkaline phosphatase (EC 3.1.3.1) linked to goat antimouse mixed immunoglobulins from Hy-Clone, *p*-nitrophenylphosphate and bovine serum albumin (BSA) from Sigma Chemical Co., polyvinyl chloride microtiter plates from Falcon Plastics, and Freund's complete and incomplete adjuvants and proteose peptone from Difco Laboratories. We obtained BALB/c mice from Charles River Laboratories, 180/TG sarcoma cell line from our laboratory collection, and potato-dextrose agar from Becton-Dickinson. Pharmacia Fine Chemicals supplied the epichlorohydrin-sucrose copolymer Ficoll 400, and we purchased ultrafiltration membranes from Millipore or Sartorius. The CU used in these studies was isolated from liquid shake cultures of the RR2 isolate and purified by the method previously reported (21,22,30).

Buffer solutions. The buffer solutions for CU-Ficoll coupling were: coupling buffer, 1 M $NaHCO_3$ and 0.5 M NaCl (pH 9.0); blocking buffer, 1 M diethanolamine in coupling buffer (pH 8.3); phosphate buffered saline (PBS), 0.01 M NaH_2PO_4, 0.001 M KH_2PO_4, 0.003 M KCl, and 0.15 M NaCl (pH 7.2).

The buffer solutions for the ELISA were: PBS-azide (PBSA), with PBS containing 0.01% NaN_3 (pH 7.2), PBSA-Tween 20, and PBS-azide containing 0.05% Tween 20; conjugation buffer, 0.01 M tris(hydroxymethyl)aminomethane, 0.15 M NaCl, and 1% BSA (pH 7.2); blocker, conjugation buffer containing 0.05% Tween 20; diethanolamine buffer, 1 M diethanolamine, 0.5 mM $MgCl_2$, and 0.01% NaN_3 (pH 9.8).

Test microorganisms. Isolates of *C. ulmi* were provided by C. M. Brasier (British Forestry Commission, Forest Research Station, Alice Holt Lodge, Farnham, Surrey, United Kingdom), D. R. Houston (Forest Service Laboratory, USDA, Hampden, CT 06518), G. A. Strobel (Department of Plant Pathology, Montana State University, Bozeman 55717), L. Miltempherger (Universita' Degli Studi, Instituto de Pathologia, Forestale e Agaria, Florence, Italy), and T. E. Hinds (United States Forest Service Laboratory,

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Fort Collins, CO 80523). Hinds also supplied cultures of *C. fimbriata* Ell. & Halst., *C. populina* Hinds & Davids., *C. piceae* (Münch) Bakshi, *C. tremulo-aurea* Davids. & Hinds, *C. ips* (Rumbold) C. Moreau, *C. distorta* Davids., *C. olivaceapinii* Davids., *C. sagmatospora* Wright & Cain, *C. microspora* (Davids.) Davids., *C. abiocarpa* Davids., and *C. minor* (Hedge.) Hunt. Strobel also supplied a culture of *C. montia* (Rumb.) Hunt, and D. Plourde (Department of Plant Pathology, University of Massachusetts) provided a second isolate of *C. ips*.

Several bacteria isolated from elm tissue (12) were also tested with the ELISA. C. W. Murdoch (Northeast Plant, Soil and Water Laboratory, USDA/ARS, and Department of Botany and Plant Pathology, University of Maine, Orono 04469) and R. J. Campana (Department of Botany and Plant Pathology, University of Maine) supplied cultures of *Enterobacter cloacae* Jordan, *E. agglomerans* Beijerinck, *Klebsiella oxytaca* Flugge (two isolates), *Bacillus megaterium* de Bary, *Pseudomonas fluorescens* Migula, *Serratia fonticola* Gavini et al, and *Staphylococcus* spp. Plourde provided a culture of *P. syringae* van Hall plus eight cultures of other *Pseudomonas* spp. In addition, K. Furuya (Fermentation Research Laboratories, Sankyo Co. Ltd., Tokyo, Japan) sent cultures of 20 unidentified bacteria and 19 unidentified yeast, all isolated from elm.

A number of fungi not isolated from elm tissue were also screened. These included three Basidiomycetes (*Sporobolomyces* spp., *Ganoderma lucidum* (Leyss.: Fr.) P. Karsten, and *Flammulina velutipes* (Fr.) P. Karsten), two Ascomycetes (*Penicillium melinii* Thom and *Saccharomyces cerevisiae* Meyen ex Hansen), and one Zygomycetes (*Mucor rouxii* Calmette) supplied by H. E. Bigelow (Department of Botany, University of Massachusetts) and one Deuteromycetes (*Aspergillus awamori* Nakasawa) from the collection of J.H.N.

Preparation of plate cultures. Plate cultures were used to screen microorganisms. Unless otherwise noted, fungal plate cultures (excluding yeast) used for analysis of CU or immunological cross-reactive material were grown on 4% potato-dextrose agar and bacteria and yeast were grown on 4% trypticase soy agar. Petri plates (50 mm diam.) containing an appropriate medium were inoculated and incubated at 25 C in the dark until the colony covered the entire agar surface of the plate. The growth period varied with each organism.

Plate cultures were extracted employing a modification of the procedure of Takai and Hiratsuka (27). One milliliter of sterile water was added to the plate, and the surface of the colony was rubbed gently with a bent glass rod, with care taken not to disturb the agar. More concentrated extracts were prepared by transferring the first extract with a Pasteur pipette to a duplicate colony and repeating the process. Extracts were added to 1.5-ml Eppendorf microfuge tubes and the volumes made to 1 ml with water. Insoluble debris was removed by centrifugation in an Eppendorf centrifuge at 15,000 *g* for 3 min at room temperature, and the supernatants were collected and stored frozen until use. The solubility of CU has been shown to be high in 70% ethanol (16). However, since extracts of plate cultures of five different *C. ulmi* isolates with water yielded the same results as obtained with 70% ethanol, water was chosen for extraction.

Preparation of liquid cultures. To obtain more accurate information on CU production by *C. ulmi* isolates, liquid shake cultures were used. *C. ulmi* isolates were grown as described previously (25,30) except that three 125-ml Erlenmeyer flasks, each inoculated with 25 ml of medium, were used. The cultures were then pooled and centrifuged at 1,465 *g* for 30 min at 4 C to remove vegetative cells. Culture filtrate was filtered through a 0.45-μm membrane, and the volume was adjusted with water to 75 ml for the turbidimetry assay. Total culture filtrate, including the sample used in turbidimetry, was then freeze-dried. Residues were dissolved in 1 ml of distilled water, and the quantity of CU in each sample was determined on serial dilutions by the ELISA.

Antigen preparation. CU purified as described elsewhere (21) was judged homogenous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (9). Because of its relatively small size (M_r 13,500), however, CU was regarded as a potentially weak immunogen. Therefore, two forms, either free CU or CU coupled to cyanogen bromide (CNBr)-activated Ficoll 400, were used as immunogen. Ficoll was activated with CNBr by the method of March et al (11). The reaction mixture (5.5 ml) consisted of 10 mg of purified CU in 3 ml of coupling buffer added to 2.5 ml of a CNBr-activated Ficoll suspension that originated from 50 mg of activated Ficoll. The mixture was stirred (31) overnight and dialyzed against water; any free functional groups were inactivated with blocking buffer. After dialysis against water, uncoupled CU was separated from the product by filtration through a CFSA filter (Amicon). The CU-Ficoll complex was triturated with $(NH_4)_2SO_4$ to 60% of saturation, and the precipitate was dissolved in PBS. After dialysis against PBS at 4 C, the CU-Ficoll was stored at 4 C.

Immunizations. Female BALB/c mice 6–8 wk old were used as the source of antibodies. Before immunization, approximately 150 μl of preimmune (control) blood was obtained from tail veins. Clot-free serum was made 0.01% in NaN_3 and stored at 4 C. A 300-μl solution of CU (0.55 mg/ml) or a suspension of CU-Ficoll (1 mg CU/ml of packed beads), both in sterile PBS, was emulsified with an equal volume of Freund's complete adjuvant. A 200-μl aliquot of the emulsion, containing either 55 μg of CU or 100 μg of the CU conjugate, was injected into the intraperitoneal cavity of each mouse. Each animal received two booster injections of the same antigen emulsified in Freund's incomplete adjuvant—the first 4 wk after the primary injection and the second about 1 wk later. Development of antibodies in the immunized mice was monitored by ELISA performed on blood samples collected 7 days after the second booster injection.

Ascitic fluid. BALB/c mice immunized with CU were used to produce ascitic fluid by the procedure of Sartorelli et al (19). Each was given a final booster injection of 55 μg of CU. Two days later, each mouse received an intraperitioneal injection of approximately 3.0×10^{10} 180/TG sarcoma cells in a total volume of 250 μl of sterile PBS. When abdominal distention became pronounced (about 10 days), the mice were anesthetized and the ascitic fluid (usually 3–5 ml) was collected by inserting an 18-gauge hypodermic needle into the intraperitoneal cavity and collecting the fluid in a 10-ml syringe. Cell debris was removed by centrifugation at 1,500 *g* for 10 min at room temperature. The ascitic fluid was kept at 4 C overnight, and the fatty layer that formed on the surface was removed the next day. The fluid was made 0.01% in NaN_3 and stored at 4 C. Because immunization with either free CU or CU-Ficoll conjugate produced equivalent antibody titers, all experiments reported here were conducted with ascitic fluids from mice immunized with free CU. Ascitic fluid from mice immunized with *Salmonella typhimurium* flagellin served as a control.

ELISA. The indirect ELISA format was applied as described by Voller et al (33). For assay of plate cultures, microtiter wells containing 200 μl of extracts were incubated in duplicate overnight in a drying oven at 40 C. All solvent evaporated, leaving the wells coated with antigen. This procedure gave higher spectrophotometric readings and reproducibility than incubating solutions in the wells. With culture filtrates, however, coating the wells by evaporation was precluded because the dissolved solids left a residue that severely depressed the ELISA color. (The small diminution in color yield from not evaporating was more than compensated for by not having residue in the wells.) With filtrates from liquid cultures, therefore, wells contained duplicate fivefold dilution series of 25 μl of each culture filtrate in 100 μl of water. Microtiter plates were covered to prevent evaporation and incubated overnight at room temperature. Except for differences in the antigen coating procedure, the assay protocol for both plate extracts and culture filtrates was the same, with all subsequent steps conducted at room temperature.

Wells were washed three times with PBSA-Tween 20, filled with blocker, and incubated for 30 min. Plates were washed as previously described and incubated overnight with 100 μl of appropriately diluted anti-CU antibody preparations (sera or ascitic fluid). After three washings with PBSA, 100 μl of goat antimouse immunoglobulin conjugated to alkaline phosphatase (diluted 1:1,000 in conjugation buffer) was added to each well, and the mixtures were incubated for 2 hr. After three washings with

PBSA-Tween 20, 100 μl of 10 mM *p*-nitrophenylphosphate in diethanolamine buffer was added and the mixtures were incubated until the well containing 1,000 ng of standard CU gave an absorbance (*A*) of approximately 0.9 at 405 nm with a Biotec ELISA plate reader. Uncoated wells and wells coated with antigen but without anti-CU antibodies served as negative controls.

To ascertain an appropriate concentration of anti-CU antibody, ascitic fluids were serially diluted in conjugation buffer and analyzed by the ELISA with 100 ng of CU in each well. Similar tests with sera were also used to monitor the titer of circulating antibodies in immunized mice before injection with sarcoma cells.

In all assays, a set of standard CU solutions in 70% ethanol ranging in concentration from 0.05 to 50 ng/μl was employed. Twenty-microliter aliquots of each of these stock standards were added in duplicate to wells containing 100 μl of distilled water; 20 μl of 70% ethanol was added to blank wells. The solutions were incubated overnight at 40 C to evaporate all of the solvent, and CU concentrations in extracts or filtrates were then determined from the standard curves.

Turbidimetry. Turbidimetry has been used to evaluate the levels of CU in culture filtrates of *C. ulmi*. From these values, the CU production index (CPI) was determined (26,30).

RESULTS

Serum titers of mice immunized with either CU or the CU-Ficoll conjugate were approximately equivalent, with dilutions of 5×10^2 to 1×10^3 providing one-half maximal A_{405nm} values (Fig. 1) after 45–60 min of incubation in the ELISA described (data not shown). Similarly, a 1–10 dilution of ascitic fluids from mice immunized with either CU or the CU-Ficoll conjugate gave one-half maximal readings during the same period (data not shown).

Preimmune serum as well as serum or ascitic fluids from mice immunized with flagellin of *S. typhimurium* were unreactive in the ELISA in either the presence or the absence of purified CU or with extracts of *C. ulmi* or a test panel of yeast, fungi, and bacteria selected from among the organisms examined (data not shown).

Figure 1 shows a typical standard curve of A_{405nm} plotted vs.the log of CU concentration. Quantitative analyses of all samples were restricted to absorbance readings that corresponded to CU concentrations between 20 and 250 ng per well. Because the shape of the curve varied slightly from one experiment to another, a standard curve was included with each set of assays.

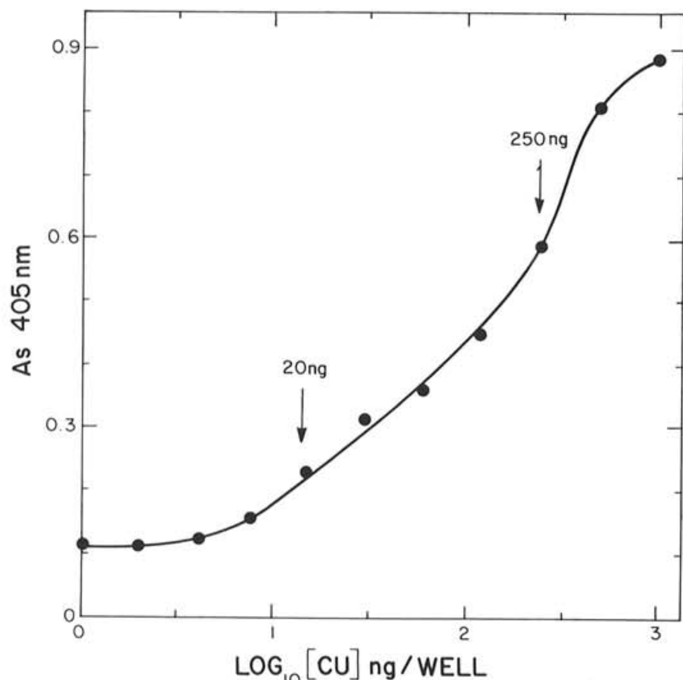

Fig. 1. Representative standard curve of absorbance values obtained at various concentrations of cerato-ulmin (CU) using the ELISA described in the text.

Although the major objective of this work was to survey a large number of *C. ulmi* isolates for CU production, the utility of these antibodies ultimately depends on specificity for *C. ulmi*. Therefore, testing of cross-reactivity was also necessary. In this context, the following were considered: including a wide taxonomic range of microorganisms, testing bacterial and yeast isolates associated with elm tissues, and surveying other members of the genus *Ceratocystis*. Thirteen members of the genus *Ceratocystis*, including 86 isolates of *C. ulmi*, were screened. Also tested were seven species of bacteria from six genera plus 29 unidentified bacterial isolates, four families of fungi that included seven genera other than *Ceratocystis*, and numerous unidentified fungal isolates. Among these, 37 bacterial and 19 yeast isolates were cultured from elm wetwood and/or sapwood.

CU production by plate cultures of the 86 *C. ulmi* isolates tested by the ELISA was divided into three groups: 0–14 ng per well, no CU production, 28 isolates; 15–27 ng per well, questionable CU production, 2 isolates; and 28 ng or more per well, CU production, 56 isolates. Extracts with less than 15 ng of CU per well were judged as negative because baseline absorbance values between 0.1 and 0.2 (0–14 ng per well) could not be distinguished from control (blank) absorbancies. The two isolates in the questionable group (15–27 ng per well) produced 22 and 25 ng per well, respectively. Of the 56 isolates producing 28 ng or more of CU per well (twice the average background absorbance), 10 produced more than 28 but less than 50 ng, 31 produced more than 50 but less than 250 ng, and 15 produced more than 250 ng (amounts of CU greater than 250 ng per well could not be quantitated because the standard curve was not accurate at high absorbance values).

Table 1 compares the results of the ELISA for CU production by 10 *C. ulmi* isolates in plate and liquid cultures with the values obtained by turbidimetry of culture filtrates. The isolates were separated into two groups of five based on CU production in the plate assay; members of the first group produced between 52 and 250 ng of CU per well. In each case, the ELISA of CU in the plate extract agreed strongly with that for the culture filtrate. Although a direct quantitative correlation between CU contents as measured by the ELISA of plate culture and culture filtrate was not always obtained (e.g., isolates 46 and 79, Table 1), it must be remembered that plate culture extracts and culture filtrates were obtained from cultures grown under different conditions at different times. CU in the plate cultures of the second group of isolates was present at background levels and results were therefore judged as negative. Also, little or no CU could be detected in these culture filtrates by either the ELISA or turbidimetry. The ELISA, as currently configured, can detect as few as 200 ng (15 picomoles) of CU per milliliter of culture filtrate.

To determine if other members of the genus *Ceratocystis* have the capacity to produce CU, we tested extracts of plate cultures of 12 species with the ELISA. None contained any material that

TABLE 1. Comparison of ELISA and turbidimetry for determining cerato-ulmin production by selected *Ceratocystis ulmi* isolates

	ELISA		
Isolate[a]	Plate culture extract (ng/well)	Culture filtrate (ng/ml filtrate)	Turbidimetry with culture filtrate (CPI[b])
77	250	17,300	2,336
50	150	12,000	1,283
46	131	16,000	696
84	103	2,130	432
79	52	9,100	1,018
96	4	0	0
129	3	0	7
95	4	0	0
113	5	0	0
P32	1	0	0

[a] All isolates supplied by C. M. Brasier.
[b] Cerato-ulmin production index = turbidity (A_{400nm}) × 100 × dilution factor of culture filtrate (25). Values are averages of three to nine replicates.

reacted with the anti-CU antibodies. We used the *C. ulmi* isolate provided by T. E. Hinds as a control, and its extract gave a strongly positive reaction (greater than 250 ng per well) in the assay. Turbidimetric and microscopic analyses of culture filtrates, based on formation of visible structures typical of CU, had previously shown that *C. piceae* and *C. minor*, in addition to *C. dryocoetidis*, Kend. & Moln., *C. fagacearum* (Bretz) Hunt, and *C. major* (von Beyma) C. Moreau, do not secrete CU (24). The number of *Ceratocystis* species that do not produce CU is therefore extended to 15. We conclude that CU is most likely a metabolite specific to *C. ulmi* and that this protein can serve as a biochemical marker for differentiating *C. ulmi* among other members of the genus.

We also examined the fungal genera, representing four subdivisions of the Amastigomycota (1), for cross-reactivity. None was observed. Therefore, no extractable metabolite that might be generally associated with these four broad groups of fungi interferes with this assay. A number of microbial species, many associated with wetwood of mature elms, can also infect the sapwood capillary liquid of xylem. We tested extracts from plate cultures of a number of them to evaluate potential for reactivity in the ELISA. Again, no cross-reactivity was detectable.

Several isolates of *C. ulmi* that were strong producers of CU on the screening medium were also grown on trypticase soy agar, and a number of bacterial isolates were grown on potato-dextrose agar. The changes of growth media did not affect in any measurable way the relative abilities to produce water-extractable material capable of reacting with the anti-CU antibodies.

DISCUSSION

The ELISA using murine antibodies to CU provides a specific, sensitive, reliable, and quantitative assay for this toxin in biological samples and for the identification of *C. ulmi*, provided the isolate produces CU. When the two methods are compared, the ELISA is highly specific for CU and suffers less from interference by components of culture filtrates. The ELISA is also about 30 times more sensitive, is fully quantitative, and is best suited for measuring minor amounts of CU. Despite some disadvantages, turbidimetry has the significant merits of simplicity and rapidity (comparable to the ELISA) in assigning isolates to positive or negative groups.

Although the number of fungi and bacteria tested was relatively large and comprehensive and many had been isolated from elm tissue, only extracts of *C. ulmi* cross-reacted with the antibodies. Apparently, the epitope(s) of CU recognized by the mouse immune system are not shared by proteins normally associated with the exterior of these fungal and bacterial cells. This apparent absence could reflect the fact that CU has unique physical and chemical properties (16,17). Because nonproducing isolates were also negative for CU production by turbidimetry, the possibility that these particular *C. ulmi* strains are synthesizing a structural variant of CU that does not have epitope(s) necessary for antibody binding is most unlikely. Therefore, the nonproducing isolates are probably mutants displaying greatly reduced synthesis or export of the toxin. An examination of these isolates for increased intracellular accumulation of CU may be helpful in differentiating these two possibilities.

Previous work by us and others has shown a strong correlation between CU production (measured turbidimetrically) and pathogenicity among wild-type *C. ulmi* isolates (6,14,26). As shown in Table 1, the ELISA and turbidimetry of culture filtrates gave an excellent quantitative correlation among isolates not producing CU. Although differences were noted between the two methods regarding the CU present in a high producer, there was complete agreement on the rating of these isolates as abundant producers of CU. The clustering of isolates into two groups (negative and abundant producers) was observed previously (25) using turbidimetry. Of the 86 isolates we tested, only two fell between these two major groups. Similarly, previous results with turbidimetry revealed such a gap exists (24). Experiments to determine what regulates CU production/secretion by *C. ulmi* and to gain further understanding of the molecular relationship between this molecule and pathogenicity are now in progress.

Although the physiological function of CU is unknown, it is an integral component of synnema head fluid of *C. ulmi* (5,29) and thus could facilitate dispersal of spores by virtue of surface-active properties. If this is true, CU might aid pathogen vectoring by the elm bark beetle, since the fungus forms synnemata and spores beneath the bark of dead elm wood and the movement of beetles in galleries in these locations results in spores being transferred to their bodies (10).

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