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Inoculum Size Effect in Dimorphic Fungi: Extracellular Control of Yeast-Mycelium Dimorphism in *Ceratocystis ulmi*

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We studied the inoculum size effect in *Ceratocystis ulmi*, the dimorphic fungus that causes Dutch elm disease. In a defined glucose-proline-salts medium, cells develop as budding yeasts when inoculated at $\geq 10^6$ spores per ml and as mycelia when inoculated at $< 10^6$ spores per ml. The inoculum size effect was not influenced by inoculum spore type, age of the spores, temperature, pH, oxygen availability, trace metals, sulfur source, phosphorous source, or the concentration of glucose or proline. Similarly, it was not influenced by added adenosine, reducing agents, methyl donors, amino sugars, fatty acids, or carbon dioxide. Instead, growing cells excreted an unknown quorum-sensing factor that caused a morphological shift from mycelia to budding yeasts. This yeast-promoting effect is abolished if it is extracted with an organic solvent such as ethyl acetate. The quorum-sensing activity acquired by the organic solvent could be added back to fresh medium in a dose-dependent fashion. The quorum-sensing activity in *C. ulmi* spent medium was specific for *C. ulmi* and had no effect on the dimorphic fungus *Candida albicans* or the photomorphogenic fungus *Penicillium isariaeforme*. In addition, farnesol, the quorum-sensing molecule produced by *C. albicans*, did not inhibit mycelial development of *C. ulmi* when present at concentrations of up to 100 μM. We conclude that the inoculum size effect is a manifestation of a quorum-sensing system that is mediated by an excreted extracellular molecule, and we suggest that quorum sensing is a general phenomenon in dimorphic fungi.

Fungal dimorphism is defined (20) as an environmentally controlled reversible interconversion of the yeast and mycelial morphologies. Interest in this phenomenon derives from the prevalence of dimorphism among those fungi exhibiting pathogenicity towards plants and animals. Numerous chemical and environmental parameters have been reported to shift the yeast-mycelium dimorphism. Among these have been temperature (18), pH (18), glucose levels (2, 3, 5, 18), nitrogen source (12, 22), carbon dioxide levels (2), and transition metals and chelating agents (3, 8, 17, 18), as well as the inoculum size or cell density employed (3, 12, 19, 24).

We have been studying quorum sensing in the regulation of yeast-mycelium dimorphism in fungi. In *Candida albicans*, we recently showed (9) that the inoculum size effect results from production of farnesol. Farnesol is continuously excreted by *C. albicans* during growth in amounts roughly proportional to the number of CFU per milliliter. At a sufficiently high level (1 to 5 μM), farnesol prevents mycelial development during growth. It also blocks germ tube formation caused by three chemically distinct triggers: L-proline, *N*-acetylglucosamine, and serum. In all cases, the presence of farnesol at concentrations of up to 250 μM prevents the yeast-to-mycelium conversion, resulting in actively budding yeasts without influencing cellular growth (9). Farnesol exhibits general cross-reactivity within *C. albicans* in that supernatants from strain A72 are active on five other strains of *C. albicans* and vice versa.

In *Ceratocystis ulmi* (12), the causative agent of Dutch elm disease, the nitrogen source controls dimorphism. At cell densities of $\geq 10^6$/ml in a defined liquid medium containing phosphate salts and either glucose or sucrose, proline (10 mM) induced the yeast morphology, while a 10 mM concentration of either ammonium, asparagine, or arginine induced the mycelial morphology (12). For both the ammonium- and arginine-containing media, inoculum size (10^3 to 10^8 blastospores per ml, final concentration) had no effect on morphology; mycelia were produced in all cases. With proline, budding yeasts formed only when the cell concentration was $\geq 10^6$ blastospores per ml. Smaller inoculum sizes produced a transient mycelial stage with the mycelium length inversely proportional to inoculum size (12). We termed this phenomenon the "inoculum size effect" (12). Throughout we will refer to the extracellular cell density-dependent signals produced by *C. ulmi* and *C. albicans* as "quorum-sensing molecules" (QSMs), in part because there is little information on the mode of action of these factors. Our objective in this study was to determine how similar the inoculum size effect in fungi is to quorum sensing in bacteria, whether dimorphic fungi other than *C. albicans* use similar signaling systems, and whether farnesol could initiate cross talk between *C. albicans* and those other fungi.

MATERIALS AND METHODS

Organism. *C. ulmi* (Buism), C. Moreau, was obtained from the National Center for Agricultural Utilization Research (NRRL 6404 and 6405) Peoria, Ill.,

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as was the photomorphogenic fungus *Penicillium isariaeforme* (NRRL 2639). *C. ulmi* also is known as *Ophiostoma ulmi*. Cultures were maintained at 5°C on potato dextrose agar (PDA) (1) slants; culture transfers to fresh PDA slants were made every 6 months.

Growth medium. The defined liquid medium contained the following per liter of distilled water: glucose (or sucrose), 20 g; KH_2PO_4, 4 g; Na_2HPO_4, 3.2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; biotin, 20 μg; thiamine · HCl, 200 μg; pyridoxine · HCl, 200 μg; $ZnSO_4 \cdot 7H_2O$, 1 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $CuSO_4 \cdot 5H_2O$, 1 mg; $FeCl_3$, 1 mg; 10 mM L-proline or 10 mM L-arginine · HCl (pH 6.0 ± 0.2). The vitamins were prepared as a 1,000× stock mixture in 20% aqueous ethanol, and the trace elements were prepared as a 5,000× stock solution (5 mg/ml in 0.1 N hydrochloric acid). The medium ingredients, except carbon and nitrogen sources, were dissolved in distilled water, dispensed into culture flasks as required, and autoclaved at 121°C for 15 min. The nitrogen sources were prepared as 100 mM stock solutions, autoclaved separately, and added aseptically to the medium to give a final concentration of 10 mM. The chemical nature of the nitrogen source was important, but the actual number of nitrogen atoms in the nitrogen source was not (12). For proline, asparagine, and arginine (one, two, and four nitrogens, respectively), cell morphology did not vary over the range from 1 to 100 mM (12). Thus, 10 mM was chosen for routine use. Similarly, glucose (or sucrose) was prepared as a 20% stock solution, autoclaved separately, and added aseptically to the growth medium to give a final concentration of 2%. (Hereafter, the abbreviations SPP, GPP, GPR, and GPA represent sucrose-phosphate-proline, glucose-phosphate-proline, glucose-phosphate-arginine, and glucose-phosphate-ammonium media, respectively.)

Preparation of inoculum. For blastospore preparation, flasks (250 ml) containing 50 ml of GPP or SPP were aseptically inoculated with cells from the sporulated stock cultures maintained on PDA slants. The cultures were incubated at 25°C on a G52 New Brunswick Scientific Co. gyratory shaker at 150 rpm for 5 days. The cells were harvested aseptically, pelleted by centrifugation for 5 min at 4,750 × g, and washed three times (strain 6404) or six times (strain 6405) with an equal volume of sterile 50 mM phosphate buffer (pH 6.5). Cells were resuspended in 10 ml of the same buffer and stored at 5°C until needed as blastospore inocula. These inocula were composed of approximately 60% blastospores and 40% yeast-phase cells. For the conidiospore preparation, blastospores were inoculated into 250-ml flasks containing 50 ml of GPR medium to a final cell concentration of 2 × 10⁷ cells per ml. The flasks were incubated at 25°C on a G52 shaker at 150 rpm for 4 to 6 days. Conidiospores were separated from mycelial growth by filtering the culture through milk filters (Kendall Co., Boston, Mass.), which retain the mycelia. The conidiospores were pelleted from the filtrate by centrifugation for 10 min at 4,750 × g, washed twice, resuspended in 50 mM phosphate buffer (pH 6.5), and stored at 5°C.

Culture conditions. Liquid shake cultures were made in 250-ml flasks containing 50 ml of the designated growth media and incubated at 23 to 25°C. Aeration was accomplished by swirling at 150 rpm on a New Brunswick Scientific Company G52 gyratory shaker. The morphology of the culture was monitored 18 to 20 h after inoculation by making wet mounts on a glass slide and viewing the sample at ×640 by phase-contrast microscopy. Cultures with fewer than 10⁶ cells per ml were too sparse for easy microscopic observation. Therefore, 5-ml aliquots of these samples were aseptically removed and pelleted by 10 min of centrifugation at 4,750 × g before microscopic examination. At least 100 differentiated cells were counted from each sample. Only differentiated cells (filamentous or budding) are presented, and, therefore, the percentages of yeasts and filamentous cells always total 100%. Cells with buds attached were counted as yeast cells. Spores forming germ tubes were counted as germinated if the length of the germ tube was greater than half the diameter of the spore. Undifferentiated spores that had not yet undergone any morphogenetic development were also counted, but no differences were observed.

Standard experimental design with various inoculum sizes. Cell density was determined by counting spores in a hemacytometer. If yeast-phase cells were desired, the standard inoculum size in GPP medium was 2 × 10⁷ cells/ml. To obtain lower initial cell densities, serial dilutions were made in sterile 50 mM phosphate buffer. Inoculated flasks were incubated for 24 h at 22 to 25°C with shaking at 150 rpm, after which cell morphology was determined by phase-contrast microscopy. All measurements were conducted in triplicate. In this system, the first visible buds or germ tubes appear 18 to 24 h after inoculation.

Spent media. The spent media were generated by inoculating 50 ml of GPP or GPR medium in 250-ml flasks with 10⁷ CFU of either *C. ulmi* (conidiospores) or *C. albicans* A72 per ml. Flasks were aerated by rotary agitation at 150 rpm on a New Brunswick Scientific Co. G52 shaker for either 60 to 72 h (*C. ulmi*) or 20 to 24 h (*C. albicans*). These incubation times gave equivalent cell densities. In all cases, the pH after incubation was 5.7 ± 0.1. The cultures were then harvested

by centrifugation (10 min at 4,750 × g) and sterilized by passage through 0.45-μm-cutoff filters.

Other methods. Elevated levels of CO_2 (5, 10, and 15%) were achieved in 250-ml flasks with two-hole rubber stoppers. Gas flow was controlled with two flow meters—one connected to an air pump and the other connected to a tank of 100% CO_2. A Y-connector combined the two flows. For instance, 15% CO_2 had a CO_2 flow of 15 and an airflow of 85. The mixed gases were filter sterilized and flushed through the growth flask for 20 min. The "gas-in" and "gas-out" lines were then clamped off, and the flasks were placed on a New Brunswick Scientific Co. G52 shaker for 24 h. The effect of spore age on conidiospore and blastospore inocula was determined by growing *C. ulmi* in Fernbach flasks containing 500 ml of GPR or GPP medium, respectively. Beginning on day 4, aliquots (50 ml) were removed aseptically from the flasks for spore preparation. All chemical supplements were either autoclaved separately or filter sterilized separately prior to aseptic addition. All organic solvent extraction protocols were performed as described previously (9).

RESULTS

Inoculum size effect. In proline-containing media (SPP or GPP), budding yeasts of *C. ulmi* occurred only with inocula giving final cell densities of ≥10⁶ spores per ml (Table 1). Morphology was independent of inoculum spore type (i.e., blastospores versus conidiospores) or carbon source (i.e., sucrose or glucose).

TABLE 1. Effect of inoculum size on cell morphology in *C. ulmi* at 20 h of growth

Inoculum size cells/ml	% of mycelia with growth on[a]:	
	GPP	GPR
2 × 10⁷	16	95
1 × 10⁷	15	95
8 × 10⁶	20	95
6 × 10⁶	20	95
4 × 10⁶	20	95
2 × 10⁶	18	95
1 × 10⁶	27	95
4 × 10⁵	95	95
1 × 10⁵	95	95
4 × 10⁴	95	95

[a] Filamentous growth is given. Only differentiated cells were counted; thus, the percentages of mycelial-filamentous growth and percentage of yeasts sum as 100%. GPP medium contains proline as the nitrogen source, whereas GPR medium contains arginine. Values shown are averages of data from triplicate experiments performed earlier (14). Similar results agreeing with ±10% were obtained by four researchers using two strains (6404 and 6405) over a span of 20 years.

TABLE 2. Variables that did not affect the inoculum size effect in *C. ulmi* at 20 h of growth

Variable	Range
Temp (°C)	15–29[a]
pH	3–7
Glucose concn (%)	0.4–8
Sucrose concn (%)	0.4–8[b]
Proline concn (mM)	0–100
Phosphate concn (mM)	5–500
Spore age (days)	0–10
Atmospheric CO_2 (%)	0–15%

[a] Note that 32°C is the maximum temperature at which these strains of *C. ulmi* will grow (11).
[b] For *C. ulmi*, glucose and sucrose are interchangeable in terms of all growth parameters (11, 14).

TABLE 3. Dose response of QSM activity in *C. ulmi* at
20 h of culture

Vol of extract (μl of methanol)	% of mycelia[a]
0	93 ± 6
2	62 ± 3
5	58 ± 10
10	43 ± 9
20	38 ± 6
50 (methanol control)	91 ± 3

[a] Filamentous growth is given as the average of data from triplicate experiments ± standard error.

Eliminating alternatives. The unifying theme for the variations chosen is that they reflect additions and/or treatments known to shift the yeast/mycelium ratio in other dimorphic fungi. In GPP medium, all of the variations shown in Table 2 did not shift the mycelium-yeast threshold of 10^6 spores per ml normally observed. Spore age was examined, because many fungal spores exhibit constitutive dormancy (6, 15, 16) and will not germinate except with the passage of time. For instance, in our study of chlamydospores from *C. ulmi* (15), we did not detect spore germination until 6 months after spore formation.

Additionally, the inoculum size threshold in GPP was not shifted by any of a series of chemical additions. The additives tested included adenosine (up to 0.1%), EDTA or EGTA (up to 0.1%), a series of fatty acids, amino sugars, and potential methyl donors, as well as the enzymes catalase and cyclic AMP (cAMP) phosphodiesterase. None of these additions shifted the normal inoculum size threshold in GPP medium. The inactivity of added catalse and cAMP phosphodiesterase precluded QSM activity by hydrogen peroxide or cAMP, respectively. Each of these chemical additions was chosen based on published reports that it altered yeast-mycelium dimorphism in at least one dimorphic fungus (11, 12, 14).

QSM in spent media. *C. ulmi* could produce extracellular molecules whose concentration determines whether development is in the yeast or mycelial mode. This hypothesis was supported by including filter-sterilized spent medium in fresh SPP medium. At 10^5 spores per ml, the inclusion of spent growth media caused a progressive, dose-dependent shift from mycelia to yeasts. The percentage of mycelia decreased from 91% to 79, 57, and 25% with the addition of 0, 12, 50, and 66% (vol/vol) spent medium, respectively. The unknown QSM from *C. ulmi* was not highly volatile, because the inoculum size threshold remained unchanged when *C. ulmi* was grown in sealed 250-ml center well flasks with and without activated charcoal in the center well.

QSM activity is lipophilic. *C. ulmi* spent medium was extracted with ethyl acetate, whereupon the ethyl acetate was separated and dried by rotary evaporation. The residue was resuspended in 100% methanol and bioassayed for QSM activity (Table 3). Addition of methanic or QSM decreased mycelium production in a dose-dependent fashion, and in each case, the morphological shift was from mycelia to actively budding yeast cells. The solvent control (50 μl of methanol, representing 1% of total volume) had no effect on normal mycelial growth (Table 3). On a quantitative basis, 60% of the QSM activity originally present in the spent medium was recovered

in the methanol. This value is reasonable in view of probable losses during drying. As expected, the extraction of QSM activity into the organic solvent was accompanied by its disappearance from the spent medium. Following ethyl acetate extraction, the aqueous phase was further extracted with hexane (to remove residual ethyl acetate) and then bioassayed for QSM activity. No QSM activity was detected; all values for mycelia were within ±5% of those obtained with unsupplemented SPP medium. Hexane extraction of the residual ethyl acetate was necessary because trace levels of ethyl acetate are inhibitory for *C. ulmi*.

QSM activity is specific to *C. ulmi*. The QSMs from *C. albicans*, *C. ulmi*, and *P. isariaeforme* appear to be distinct from one another, since these fungi respond only to their own spent media. At 10^5 spores per ml, *C. ulmi* formed ≥80% mycelia in unsupplemented GPP medium or GPP medium supplemented (84% by volume) with spent medium from *C. albicans* or *P. isariaeforme*. However, *C. ulmi* formed only 15 to 20% mycelia when supplemented with its own spent medium. Also, *C. ulmi*'s spent medium did not affect the growth of *C. albicans*. At 37°C, *C. albicans* formed ≥80% mycelia in regular, unsupplemented germ tube differeniation assays (9) or in assays supplemented (80% by volume) with spent medium from *C. ulmi* or *P. isariaeforme*. In these assays, *C. albicans* only formed 10 to 15% mycelia when supplemented with its own spent medium.

Further evidence that the QSM from *C. ulmi* is distinct from farnesol, the QSM for *C. albicans* (9), is provided by its distinct smell, its stability in methanol, and the fact that *E,E*-farnesol (0, 1, 5, 10, 50, and 100 μM) had no effect on yeast-mycelium dimorphism in *C. ulmi*. Similarly, geranylgeraniol (0, 17, 85, 170, and 850 μM in dimethyl sulfoxide) had no effect on dimorphism in *C. ulmi*. For comparison, with *C. albicans*, 1.2 μM *E,E*-farnesol gave 50% inhibition of germ tube formation (23).

DISCUSSION

We have shown that the inoculum size effect in *C. ulmi*, a dimorphic fungus, is due to an extracellular, lipophilic molecule. By analogy to our previous work with *C. albicans* (9), we refer to this molecule as a QSM. When spent medium from *C. ulmi* was extracted with an organic solvent, such as ethyl acetate, all of the QSM activity was transferred from the aqueous to the organic phase. This QSM activity was specific for *C. ulmi*; it did not cross-react with *C. albicans*. QSMs appear to be organized into families of chemically related molecules. Those for gram-negative bacteria are variations on homoserine lactones (4), while those for gram-positive bacteria are peptides (10). Our results so far are consistent with the suggestion that the QSMs for dimorphic fungi are lipophilic isoprenes (9). Of course, the confirmation of this suggestion awaits actual structure determination for the QSMs from *C. ulmi* and other dimorphic fungi.

Among the small organic molecules used by nature, the isoprenoids are extraordinarily diverse in structure and function. Over 23,000 individual isoprenoid compounds have been characterized (21), and one of these, the sesquiterpene farnesol, has been identified as the QSM for *C. albicans* (9). With regard to the chemical structure of *C. ulmi*'s QSM, we note that 13 cyclic and acyclic sesquiterpenes are produced by various members of the *Ophiostoma/Ceratocystis* family (7) and

that so far the biological significance of these volatile isoprene compounds remains unknown (7). We suggest that their biological significance may be to mediate inoculum size-dependent control of dimorphism in *C. ulmi*.

Another cell density-dependent phenomenon in mycology is self-inhibition of fungal spore germination. Many fungal spores exhibit a crowding effect (6, 13) in which the spores contain a prepackaged self-inhibitor that prevents germination under crowded, high-cell-density conditions. These self-inhibitors usually are phenolic molecules, such as *cis*-ferulic acid methyl ester or *cis*-3,4-dimethoxycinnamic acid methyl ester, which can be removed by repeated washing. Although there may be some overlap between self-inhibitors and the inoculum size effect (QSMs), the two phenomena are likely distinct, because the self-inhibitors are associated with spore formation and spore germination, whereas QSMs and the morphological change they induce are growth associated. Additionally, the possibility of similar self-inhibitors in *C. ulmi* blastospores and conidiospores is unlikely, because germ tube formation in both GPA and GPR media is not cell density dependent (Table 1).

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