Tyrosol is a quorum-sensing molecule in Candida albicans

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Contributed by Gerald R. Fink, February 27, 2004

The human fungal pathogen Candida albicans shows a significant lag in growth when diluted into fresh minimal medium. This lag is abolished by the addition of conditioned medium from a high-density culture. The active component of conditioned medium is tyrosol, which is released into the medium continuously during growth. Under conditions permissive for germ-tube formation, tyrosol stimulates the formation of these filamentous protrusions. Because germ-tube formation is inhibited by farnesol, another quorum-sensing molecule, this process must be under complex positive and negative control by environmental conditions. The identification of tyrosol as an autoregulatory molecule has important implications on the dynamics of growth and morphogenesis in Candida.

Cells monitor their population density by releasing signaling molecules to which they respond. In bacteria, these auto-stimulatory compounds are usually small molecules, the concentration of which increases as the number of bacteria increases. After reaching a threshold concentration, these quorum-sensing molecules induce the population to cooperate in diverse behaviors such as bioluminescence, antibiotic production, virulence, biofilm formation, competence, sporulation, etc. (1). In Gram-negative bacteria, these compounds are often acyl homoserine lactones, and in Gram-positive bacteria, they are usually modified peptides (2).

One of the most striking quorum-sensing behaviors is the extremely long lag time shown by cells when they are diluted into fresh medium. The greater the dilution, the longer it takes for cells to initiate exponential growth. This “lag phase” is usually attributed to the requirement for autostimulatory compounds that are released into the medium as the cells proliferate but fall below some critical concentration when the cells are diluted into fresh medium. Compounds have been isolated from bacteria that shorten the lag time (3–5). Thus far, the phenomenon has not been investigated in fungi (6).

Candida albicans, a serious fungal pathogen (7–9), displays several density-dependent phenomena. After dilution, it has a long delay before resuming exponential growth. In addition, cell density controls the morphological switch between the cellular yeast form and the filamentous hyphal form: at high cell densities, Candida is in the yeast form; at low cell densities, the yeast-form cell develops a filamentous protrusion known as a germ tube (10, 11). Recently, farnesol and farnesoic acid have been identified as quorum-sensing molecules that block the morphogenesis of Candida albicans at high cell densities in C. albicans (11, 12).

In this report, we show that the delay in growth after dilution is abolished by conditioned medium (CM). The active compound in the CM is tyrosol, which is released into the medium continuously during growth. In dilute cultures, tyrosol accelerates the formation of germ tubes. These results suggest that the morphogenesis of C. albicans is under complex positive and negative control by environmental conditions.

Materials and Methods

Growth Assay. C. albicans wild-type strain SC5314 was cultured in synthetic minimum medium (SD, pH 4.3) (13) at 30°C for 24 h. Cells were collected by centrifugation, washed twice with fresh SD (pH 4.3) (13), and reinoculated at indicated cell density in either SD or SD plus compounds. Cultures were allowed to grow at 30°C. Samples were taken at indicated time points, and cell densities were measured by either counting colony-forming units or measuring the OD absorption at 600 nm. For cultures at low cell densities (10^5 and 10^3 cells per ml), cells were concentrated by a filter unit (Millipore) before measuring OD absorption. The chemically synthesized tyrosol (Fluka) was purified by reverse-phase HPLC before being used in the assays.

Activity Purification. CM was prepared from overnight cultures (24 h at 30°C) by centrifugation and filtration to remove Candida cells. For analytical scale purification, 100 ml of CM was loaded to a C18 Sep-Pak cartridge (Waters). The column was washed with H_2O and 5% acetonitrile, respectively. The active component was eluted by 15% acetonitrile. The active fraction was lyophilized and purified further by C18 reverse-phase HPLC (column: Discovery C18, 25 cm × 4.6 mm, 5 mm; gradient: 6–15% acetonitrile in 60 min; flow rate: 1 ml/min). For preparatory scale purification, 10 liters of CM was loaded to 100 g of C18 resin (Waters) and washed extensively with H_2O and 5% acetonitrile. The active compound was eluted with 15% acetonitrile, lyophilized, and fractionated by a G25 resin (Bio-Rad). The final purification was achieved by C18 reverse-phase HPLC (column: Supelcosil LC-18, 25 cm × 10 mm, 5 mm; gradient: 9–15% acetonitrile in 60 min; flow rate: 2 ml/min).

NMR Measurements. NMR spectra were recorded on a Varian AS500 NMR spectrometer at 500 MHz for 1H and 125 MHz for 13C. Data acquisition and processing were performed by using Varian VNMRS 6.1 software according to the instructions. Chemical shifts of 1H and 13C NMR spectra were referenced to the solvent peaks: 2.49 (δH) and 39.7 (δC) for DMSO-d6 and 4.80 (δH) for H_2O.

Filamentation Assay. C. albicans SC5314 was inoculated in SD (pH 4.3) at a density of 1 × 10^6 cells per ml and incubated at 30°C for 24 h. Cells were collected by centrifugation and washed twice with distilled H_2O and re inoculated at 10^5 cells per ml in prewarmed SD (pH 7.0) in the presence or absence of 20 μM tyrosol. Cultures were incubated at 37°C with shaking (200 rpm in a G10 Gyrotry Shaker, New Brunswick Scientific). Samples were taken at indicated time points and concentrated by 2,000-fold by filtration and centrifugation. The morphology of Candida cells was assessed directly by microscopy analysis.

Abbreviations: CM, conditioned medium; COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; SD, synthetic minimum medium.

Data deposition: The CaEDT1 sequence reported in this paper has been deposited in the GenBank database (accession no. AY148877).

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Gene-Expression Analysis. *C. albicans* SC5314 was cultured in SD (pH 4.3) at 30°C for 24 h. Cells were collected by centrifugation, washed twice with fresh SD (pH 4.3), and reinoculated at 10⁸ cells per ml in SD (pH 4.3), 10⁷ cells per ml in SD (pH 4.3), and 10⁵ cells per ml in SD (pH 4.3) in the presence of 20 μM tyrosol. Samples were incubated for 60 min at 30°C. Cells were collected by filtration and stored at -110°C. *Candida* total RNA was prepared and used to amplify mRNA as described (14). The amplified mRNA was reverse-transcribed in the presence of amino-allyl dUTP and subsequently labeled with either Cy3 or Cy5 fluorescence dyes (Promega) (15). The Cy3- or Cy5-labeled cRNA was used to hybridize an oligo-based *Candida* genome array (Qiagen, Valencia, CA). Microarray raw data were extracted, normalized, and filtered to remove any genes with expression that did not change at least 2-fold in the experiment. Only genes showing a consistent pattern in two experimental duplicates were considered.

RT-PCR analysis was performed by using a Sensiscript RT kit according to the manufacturer instructions (Qiagen). The sensorspecific probes for Northern analysis were amplified by PCR and labeled by random priming (Stratagene). The DNA sequences for these genes are available at http://www.genolist.pasteur.fr/CandidaDB/index.html. The primers used for PCR amplifications were ATGTGAAGGTAAATTTGAAGAAG and CTACTCATCATCATCGAATTTTGG (CaPol30), ATGCTGTCCAGATCAGCCAG and GTGGTTATTGACCTCTGT (CaPol1), ATGTCCAATTTTGTATCATCACC and GTATGTAACAAGCTTGGTGC (CaRNA21), ATGTCAGGTGGTAAAGGTAAAG and CTACAATTCTTGAGAAGC (CaHTA1), and CAAGCTTGAGGTCGTCAAGTACCTGCAAGAG and GACTAAGCTTACACCAACTCTC AAAACGTTC (CaCDC46).
AAAGAAGACAATTTAACAC and CGGATCCGCATTTC-CTCTATGGTGG (CaEDT1).

**Results**

To determine the effect of cell density on the growth of *C. albicans*, we diluted an overnight culture of the organism into fresh minimal medium (SD, 30°C, pH 4.3). When inoculated at low cell densities in SD, *Candida* went through a distinct lag phase before cells initiated exponential growth. The length of the lag phase increased dramatically with decreasing cell density (Fig. 1). In SD and at a density of $10^7$ cells per ml, *C. albicans* resumed exponential growth with no apparent lag (Fig. 1A). However, at lower densities, the lag phase was significantly longer: at a density of $5 \times 10^6$ cells per ml, they manifested a lag phase of 80 min (Fig. 1B); at $5 \times 10^5$ cells per ml, the lag phase was $\sim 5$ h (Fig. 1C).

The addition of cell-free supernatant (CM) from high-density cultures ($10^8$ cells per ml) to cells at low densities completely abolished their lag phase; in the presence of CM, even dilute cells ($5 \times 10^3$ cells per ml) underwent continuous exponential growth (Fig. 1B and C). In contrast, the CM prepared from low-density cultures had no effect on the duration of the lag phase. These observations suggest that the CM of high-density cultures contains an activity that permits highly diluted cultures to resume exponential growth without a substantial lag. Furthermore, the activity was specific to the lag phase and had no effect on the exponential growth of *C. albicans* (Fig. 1A).

The ability of the CM to attenuate the lag phase provided the assay for an activity-directed purification of the molecule. In the presence of CM a dilute culture ($10^3$ cells per ml) became turbid in 24 h, whereas in its absence it did not. The purification involved size fractionation and reverse-phase HPLC chromatography (Fig. 2A). The structure of the purified molecule was solved by measuring one-dimensional NMR spectra ($^1$H and $^{13}$C).

**Fig. 3.** The accumulation of tyrosol in the medium increases with increasing cell density. An overnight Candida culture was diluted to $1 \times 10^5$ cells per ml in SD (pH 4.3), and its growth (squares) was measured by counting colony-forming units. The yield of tyrosol at specific time points (triangles) was obtained by measuring its concentration in the medium by analytical scale reverse-phase HPLC.

**Fig. 4.** Tyrosol promotes the germ-tube formation in *C. albicans*. (A) Microscopy analysis of the morphogenesis of Candida in the presence or absence of tyrosol. Diluted Candida cultures ($1 \times 10^4$ cells per ml) were incubated in SD (pH 7) at 37°C in the presence or absence of tyrosol (20 μM) and photographed at a magnification of $\times 400$. At 1 h in SD (pH 7) without tyrosol, $\sim 5\%$ of cells formed germ tubes, whereas in the presence of tyrosol, $\sim 55\%$ of cells formed germ tubes. At 2 h, only $\sim 15\%$ of cells formed germ tubes when tyrosol was absent, whereas $\sim 80\%$ of cells formed germ tubes when tyrosol was present. (Scale bar, 25 μm.) (B) Quantitation of the germ-tube formation in Candida when diluted to $10^4$ cells per ml (percentage).
CM prepared from increasing cell density (Fig. 3). The concentrations of tyrosol in the yeast form, the suppressive effects of farnesol pre- 

covery. Presumably, at high cell densities, at which the majority of cells 

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tube.

Tyrosol also accelerates the morphological conversion of 

Codida yeast-form cells to filaments. After dilution into per- 

missive medium, the yeast-form cell develops a filamentous 

protrusion known as a germ tube (10). Standard minimal medium can be made permissive for germ-tube formation if the 

pH is raised from 4.3 to 7 and the cells are grown at 37°C. When diluted in SD (pH 7, \( \geq 10^7 \) cells per ml), \( \sim 5\% \) and \( \sim 15\% \) of cells formed a germ tube after 1 and 2 h, respectively, whereas in the presence of tyrosol, \( \sim 55\% \) and \( \sim 80\% \) of cells formed a germ tube (Fig. 4A). After 6 h, the majority of the cells formed a germ tube even in the absence of tyrosol (Fig. 4B). At higher cell densities (\( \geq 10^8 \) cells per ml) in this medium, most of the cells form germ tubes with or without tyrosol. Moreover, tyrosol does not induce germ tubes in cells grown in noninducing conditions. These observations suggest that under conditions favorable for germ-tube formation, dilute cells grown without tyrosol take longer to initiate growth and therefore the formation of a germ tube.

Previous work has shown that germ-tube formation is suppressed by farnesol, which accumulates in the medium as the cells proliferate (11, 12). Therefore, germ-tube formation in Candida is under the control of at least two quorum-sensing molecules: farnesol, which inhibits formation, and tyrosol, which promotes it. Presumably, at high cell densities, at which the majority of cells are in the yeast form, the suppressive effects of farnesol predominate.

Although the annotation and assembly of the Candida genome is incomplete, it was possible to identify genes with transcript levels that were regulated by tyrosol using whole-genome arrays (15, 19, 20). Genes with expression that is regulated by tyrosol were identified by comparing gene-expression profiles of Candida cells grown to high density (\( 10^8 \) cells per ml) with those at low density (\( 10^5 \) cells per ml) with or without tyrosol, respectively. The data were analyzed to identify those genes with expression that increased or decreased in proportion to cell density and levels that were affected by tyrosol at low cell density. The known genes with transcripts that showed the greatest reduction after dilution but no reduction when tyrosol was present are those encoding proteins involved in DNA synthesis and cell-cycle regulation (Fig. 5A). This group includes the genes encoding DNA-dependent DNA polymerase, DNA-replication factors, ribonucleotide reductases, cell-cycle regulators, DNA-repair enzymes, and chromosome-segregation factors. The expression analysis also identified a key regulator of filamentation in Candida, CaEDT1 (Q.F., H.C., B. Guo, and
cultures. If dilution causes rapid degradation of the transcripts functions critical for cell division, are destabilized in dilute the delay in growth is that these transcripts, which encode machinery and cell-cycle-control proteins. One explanation for among them are transcripts encoding the DNA-replication transcripts with abundance that is reduced rapidly after dilution. satisfied by the addition of exogenous tyrosol.

The transcriptional profiling data have identified a number of transcripts with abundance that is reduced rapidly after dilution. Among them are transcripts encoding the DNA-replication machinery and cell-cycle-control proteins. One explanation for the delay in growth is that these transcripts, which encode functions critical for cell division, are destabilized in dilute cultures. If dilution causes rapid degradation of the transcripts for these functions, then tyrosol must stabilize them. Alternatively, tyrosol may be involved in the transcriptional regulation of these genes.

The stimulation of germ-tube formation by tyrosol probably is a reflection of the abolition of the lag phase. In the absence of tyrosol, the diluted Candida cells experience a delay before they initiate the growth of germ tubes. In its presence, the cells initiate growth and therefore the formation of germ tubes immediately after dilution. Previous study has identified another quorum-sensing molecule, farnesol, that inhibits the formation of germ tubes when the cultures reach high density (11). The reciprocal control of germ-tube formation by farnesol and tyrosol must reflect a complex structure of metabolic regulation in response to environmental cues that stimulates the synthesis of these regulatory molecules.

We thank M. Lorenz, G. Gordon, and T. Volkert for assistance in microarray processing and data analysis; J. Bender, M. Jin, and S. Brady for technical advice; and members of the Fink Laboratory and Clardy Laboratory for useful discussions. This work was supported by National Institutes of Health Grants GM40266 (to G.R.F.) and CA24487 (to J.C.) and Ellison Medical Foundation Senior Scholars Award ID-SS-0038-01 (to G.R.F.). G.R.F. is an American Cancer Society Professor of Genetics.
