Regulation of *Candida albicans* Morphogenesis by Fatty Acid Metabolites

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*Candida albicans* is an opportunist dimorphic fungus that inhabits various host mucosal sites. Conversion from the yeast to the hyphal form has been associated with increased virulence and mucosal invasiveness. *C. albicans* morphogenesis is regulated by multiple signals and signaling pathways. However, signals that control morphogenesis in vivo are unknown. We investigated the effects of host long chain fatty acids, eicosanoids, and bacterial short chain fatty acids on control of germination. None of the *C*₁₈ or *C*₂₀ fatty acids tested had an effect on enhancing germ tube formation (arachidonic acid, oleic acid, linolenic acid, or γ-linolenic acid). Among the different eicosanoids, both prostaglandin *E*₂ and thromboxane *B*₂ significantly enhanced serum-induced germination by *C. albicans*. Addition of antiprostaglandin or antithromboxane antibodies to serum alone inhibited germ tube formation by almost 30%, while control antibody had no effect, indicating that these eicosanoids are major morphogenic factors in the serum. Since these molecules also bind to albumin, this may also explain the hyphal transforming activity in serum that associates with albumin. Interestingly, short chain fatty acids (butyric acid), the product of lactic acid bacteria (LAB), inhibited germination. In addition, LAB culture supernatants as well as live LAB also inhibited *C. albicans* morphogenesis. Overall, these results indicate that fatty acid metabolites and fatty acid pathways can up-regulate and down-regulate germination in *C. albicans*.

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*Morphogenesis by Fatty Acid Metabolites*

*Candida albicans* inhabits various host mucosal surfaces, where it exists as both a member of the normal microflora and a potential opportunistic pathogen. *C. albicans* is a dimorphic fungus, with the ability to grow both as a yeast and as hyphae. Conversion to the hyphal form is required for virulence (40) and invasiveness (22) in vivo. Several signaling pathways regulating morphogenesis have been identified and well characterized in *C. albicans* (reviewed in reference 24). However, in vivo stimuli are still a subject of investigation. At mucosal surfaces, *C. albicans* is met by an environment dictated by the host and bacterial microflora. Both the host and bacterial microflora produce immunomodulatory fatty acid metabolites that may influence the behavior of *C. albicans*. One mechanism by which the host controls inflammatory responses is via a network of eicosanoids (20-carbon fatty acid metabolites), which include prostaglandins and leukotrienes (7, 27, 33, 34, 42). Members of the bacterial microflora also can participate in modulating local immune responses through production of short chain fatty acids (SCFA) (5, 9, 41) and even eicosanoids (43). The concept that the local fatty acid environment within the host influences virulence traits in *C. albicans* has not been examined. Therefore, the aim of these studies was to investigate the effects of fatty acids and fatty acid metabolites on *C. albicans* morphogenesis.

The observation that *C. albicans* germinates in serum was made four decades ago (4). However, the factors in serum responsible for inducing germination remain a subject of investigation. It has been suggested that serum albumin is the factor in serum involved in inducing morphogenesis (6). However, the inability of commercial preparations of albumin to induce morphogenesis prompted investigators to further explore the role of albumin. Experiments using serum from albuminemic rats demonstrate that albumin is not required for induction of morphogenesis by serum. In addition, filtering serum through a 1-kDa membrane revealed that germination-inducing activity is also found in the hydrophobic compounds in the filtrate (14). The conflicting data concerning the ability of albumin may be due to the presence of small hydrophobic compounds that bind albumin in serum, such as fatty acids and fatty acid metabolites (35). Our laboratory and others have previously reported that prostaglandin *E*₂ (PGE₂), a cyclooxygenase product of arachidonic acid involved in control of inflammatory responses, enhances *C. albicans* morphogenesis (21, 30). *C. albicans* also produces a fatty acid metabolite similar to PGE₂ that augments hyphal transformation (30). Similarly, cyclooxygenase inhibitors such as aspirin and etodolac inhibit morphogenesis (2). The latter two observations suggest the presence of an eicosanoid/oxylipin pathway in *C. albicans* that plays a role in control of germination.

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**MATERIALS AND METHODS**

*C. albicans* germ tube assay. A crystal violet-based germ tube assay was used to measure germination as previously described (1, 31, 46). *C. albicans* strain CHN1 was grown in sabouraud dextrose broth (SDB) at 22°C (room temperature) while shaking for 48 to 72 h. Samples were washed in 1× phosphate-buffered saline (PBS) and resuspended in 100% fetal bovine serum (FBS) to give a final concentration of 10⁶ yeast cells/ml. *C. albicans* diluted in FBS was then plated into a 96-well flat-bottom plate at a volume of 100 μl/well. Additions or carrier was added (10 μl), and plates were incubated at 37°C for 2 h to induce germination. Adherent germ tubes formed were fixed, and nonadherent yeast forms were removed by sequential washes with 70% ethanol and 0.25% sodium dodecyl sulfate (SDS). Plates were washed additionally two or three times with distilled water. Plates were examined microscopically to ensure removal of non-

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adherent yeast forms. Remaining germ tube forms were then stained with 0.1% crystal violet for 5 min. Plates were then washed three times with distilled water, once with 0.25% SDS, and twice with distilled water. Crystal violet that stained germ tube forms was resolubilized by adding 200 μl of isopropanol–0.04 N HCl and 50 ml of 0.25% SDS. A spectrophotometer was used to read the \( A_{590} \).

**Antiprostaglandin antibody treatment.** Prostaglandin screening antibody (Cayman Chemicals, Ann Arbor, Mich.) was resuspended in 2 ml of 1× PBS added in 10-μl amounts to wells. This broad-spectrum antibody recognizes the prostaglandins PGE\(_2\), PGI\(_2\), PGE\(_1\), PGI\(_1\), PGF\(_2\), and PGF\(_1\). This antibody does not recognize thromboxane B\(_2\) (TxB\(_2\)), leukotrienes, or free fatty acids. TxB\(_2\) antibody (Cayman Chemicals, Ann Arbor, Mich.) was also resuspended in 2 ml of 1× PBS and added in 10-μl amounts to wells. For control wells, rabbit immunoglobulin G (IgG) (BD PharMingen, San Diego, Calif.) was added at an equivalent protein concentration as an experimental antibody.

**Fatty acid treatment.** Long chain fatty acids and SCFA were resuspended or diluted in 1× PBS and brought to pH 7.0 prior to addition to *C. albicans* germ tube assay.

**LAB treatment.** The lactic acid bacteria (LAB) *Lactobacillus casei* (ATCC 393), *Lactobacillus paracasei* (ATCC 27092), and *Lactobacillus rhamnosus* GG (ATCC 53103) were grown in *Lactobacillus* deMan, Rogosa, and Sharpe (MRS) broth (Becton-Dickson Microbiology Systems, Sparks, Md.) under microaerophilic conditions (10% H\(_2\), 5% N\(_2\), 85% CO\(_2\)) at 37°C for 24 h. An equal amount of MRS broth, live lactobacilli, or *Lactobacillus* culture supernatant (100 μl) was added to each well in the *C. albicans* germ tube assay prior to incubation at 37°C, resulting in a final concentration of 50% FBS.

**Statistical analysis.** Student’s *t* test (two-tailed, unequal variance) was used to analyze the significance of differences between experimental groups. Data with a *P* value of \( \leq 0.05 \) were considered to be significant.

**RESULTS AND DISCUSSION.**

We first investigated whether unmodified fatty acids or specific eicosanoids present in serum could be largely responsible for the candidal morphogenesis activity of serum. Various long chain fatty acids were added to *C. albicans* diluted in serum. Interestingly, none had a significant effect on enhancing germ tube formation (Fig. 1). Varying the concentration of these fatty acids between 0.1 and 10 mM did not increase or decrease their ability to affect germination (data not shown). A variety of eicosanoids, including cyclooxygenase and lipoygenase products, were also tested for their ability to affect *C. albicans* morphogenesis (Fig. 2). Of the eicosanoids tested, only PGE\(_2\) and TxB\(_2\) significantly enhanced serum-mediated germination by *C. albicans* (Fig. 2).

To neutralize the prostaglandins in serum alone, polyclonal antiprostaglandin antibodies (Caymen Biochemicals) were added to cultures of *C. albicans* plus serum and changes in germ tube formation were measured (Fig. 3). Polyclonal antiprostaglandin antibodies bind to the E, D, and F series prostaglandins. They do not recognize free fatty acids, TxB\(_2\), or leukotrienes. Antiprostaglandin antibodies inhibited germ tube formation by 29%, while control antibody had no effect (Fig. 3). These data indicate that prostaglandins are major morphogenic factors in the serum and are consistent with previous studies that PGE\(_2\) can augment germ tube formation (21, 30).

TxB\(_2\) is an arachidonic acid metabolite found in serum and not recognized by the prostaglandin screening antibody (3). We therefore tested anti-TxB\(_2\) antibody in the germ tube assay to determine if we could further inhibit germination. Addition of anti-TxB2 antibody also inhibited germ tube formation by 28% (Fig. 3). However, addition of both antiprostaglandin and anti-TxB2 antibodies was not additive (Fig. 3). Since thromboxanes and prostaglandins are both found bound to albumin in the serum (15, 25, 26, 36, 37, 47), we speculate that the bioactivity we are blocking in the serum with antiprostaglandin and anti-TxB2 antibodies may be found on the same “carrier” molecule, albumin. There is a precedent for this in the literature: the hyphal transformation activity in serum copurifies with albumin, and albumin inhibits germ tube formation (30).

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**FIG. 1.** Effect of long chain fatty acids on *C. albicans* morphogenesis. Long chain fatty acids (Caymen Chemicals) or carrier (1× PBS) was added to *C. albicans* diluted in 100% FBS to a final concentration of 0.1 nM at neutral pH. Cultures were incubated at 37°C to induce germination, and germ tube formation was measured after 2 h with the crystal violet germ tube assay. Results are expressed as percent control (% control = \( A_{590} \) for experimental well/\( A_{590} \) for control well). Background absorbances (\( A_{590} \) of the well containing 100% PBS) were subtracted out. The assay was performed in triplicate and repeated two times with similar results. *, *P* < 0.05 as determined by the Student’s *t* test. AA, arachidonic acid; LNA, linolenic acid; OA, oleic acid.

**FIG. 2.** Effect of eicosanoids on *C. albicans* morphogenesis. Eicosanoids (Caymen Chemicals) or carrier (1× PBS) was added to *C. albicans* diluted in 100% FBS to a final concentration of 0.1 nM at neutral pH. Germination was measured as described in the legend to Fig. 1. Background absorbances (\( A_{590} \) of the well containing 100% PBS) were subtracted out. The assay was performed in triplicate and repeated two times with similar results. PG, prostaglandin; Tx, thromboxane; HHT, hydroxyheptadecatrienoic acid; LT, leukotriene. *, *P* < 0.05 as determined by Student’s *t* test.
with the albumin fraction, and purified albumin also contains hyphal transformation activity (14).

The ability of C. albicans to establish colonization at various mucosal surfaces is highly dependent on the presence or absence of members of the normal bacterial microflora. Antibiotic treatment often promotes increased C. albicans carriage and infection in the oral cavity, intestinal tract, and vaginal mucosa. The ability of the bacterial flora to control or prevent C. albicans colonization is due in part to competitive exclusion of favored niches. Oral bacteria including Escherichia coli and Streptococcus spp. can inhibit C. albicans morphogenesis in vitro, which indicates that the bacterial flora may produce compounds that may inhibit germination and epithelial adherence (29).

LAB are also known to inhibit C. albicans colonization of the epithelium of the gastrointestinal tract in mice and subsequent hyphal invasion and systemic infection (45). L. rhamnosus LGG is a commonly used probiotic strain with immunomodulatory activity (16, 23, 28, 32). In a mouse model of gastrointestinal candidiasis, prior inoculation with L. rhamnosus LGG reduced C. albicans levels and invasion (45). We tested whether L. rhamnosus LGG could inhibit C. albicans germ tube formation. Addition of live L. rhamnosus LGG at a 10:1 bacterium/yeast ratio significantly inhibited germ tube formation (23 to 44% inhibition) (Fig. 4a). This inhibition is not due to competitive exclusion, because LAB do not adhere to the tissue culture plastic. In addition, incubation of heat-killed bacteria did not inhibit germination, suggesting that metabolically active bacteria are required for this effect (Fig. 4b). To investigate whether a soluble product from Lactobacillus could inhibit C. albicans germ tube formation, lactobacilli were cultured under anaerobic conditions in MRS broth for 2 or 24 h and supernatants were collected and added to C. albicans diluted in 100% FBS. Supernatants from 2-h cultures of L. casei, L. paracasei, and L. rhamnosus LGG all inhibited germ tube formation (30 to 55% inhibition) (Fig. 5a). However, the addition of 24-h cultures of LAB almost completely inhibited germination (92 to 98% inhibition) (Fig. 5b), suggesting that accumulation of a soluble compound in the culture supernatant is responsible for the inhibition. The inhibitory effect of a secreted product from L. rhamnosus LGG on C. albicans germ tube formation may represent a novel probiotic effect of this strain.

Members of the normal flora such as LAB produce large quantities of biologically active SCFA. These fatty acids, which are by-products of anaerobic fermentation, possess an anti-inflammatory function (reviewed in reference 39). Therefore, examination of the effect of SCFA on morphogenesis may provide a mechanism by which LAB prevent candidal coloni-

FIG. 3. Effect of antiprostaglandin or antithromboxane antibody on C. albicans morphogenesis in FBS. Polyclonal antiprostaglandin antibody, anti-TxB2 antibody (Cayman Chemicals), control rabbit IgG (BD Pharmingen, San Diego, Calif.), or carrier (1× PBS) was added to C. albicans yeast diluted in 100% FBS. Germination was measured as described in the legend to Fig. 1. The assay was performed in triplicate and repeated two times with similar results. Anti-PG, antiprostaglandin antibody *, P < 0.05 as determined by Student’s t test.

FIG. 4. Effect of live or heat-killed LAB on C. albicans morphogenesis. Lactobacillus spp. were grown in MRS broth for 24 h under microaerophilic conditions at 37°C at neutral pH. Live or heat-killed lactobacilli diluted in MRS broth or carrier (MRS broth) were added to C. albicans diluted in 100% FBS at various ratios of LAB to C. albicans. Germination was measured as described in the legend to Fig. 1. The assay was performed in triplicate and repeated two times with similar results. *, P < 0.05 as determined by Student’s t test. CA, C. albicans.
zation. To test the effect of SCFA on *C. albicans* morphogenesis, commercially available acetic, propionic, and butyric acid (C2, C3, and C4 SCFA, respectively) were used (Fig. 6). These were all buffered to pH 7.0 to eliminate the effect of pH on morphogenesis (12). When added to *C. albicans* diluted in 100% FBS, 25 mM butyric acid (buffered to pH 7.0) significantly inhibited germ tube formation by 40%, while addition of 100 mM butyric acid almost completely abrogated germ tube formation (98% inhibition) (Fig. 6). Interestingly, butyric acid is the SCFA that is most well known for its immunomodulatory activities (10, 38, 44). The inhibitory effect of sodium butyrate on *C. albicans* has also been reported and been linked with inhibition of chitin synthesis (11, 17). SCFA are produced in large quantities by LAB, as a by-product of fermentation (19, 39). Therefore, the levels of SCFA used in the assay are physiologically relevant. Depending on the substrate used, in vitro SCFA production can range from 8 to 80 mol/100 ml within a 24-h incubation period (8). In vivo, the SCFA concentration ranges from 20 to 140 mM in the cecum and large intestine (39). Our data indicate that one mechanism by which *L. casei* LGG inhibits *C. albicans* colonization may be via inhibition of morphogenesis via butyric acid production.

Our data demonstrate that both long chain fatty acid metabolites and SCFA influence *C. albicans* morphogenesis. Among the different eicosanoids, PGE2 and TxB2 enhance germination by *C. albicans*. Since cyclooxygenase inhibitors (aspirin, indomethacin) have been reported to inhibit germination and biofilm formation (2), this suggests the presence of an eicosanoid/oxylipin pathway that plays a role in morphogenesis. This also suggests that the immune response of the host (eicosanoid microenvironment) could influence *C. albicans* morphogenesis or virulence in vivo. Interestingly, SCFA (butyric acid) inhibit germination. This may represent a novel probiotic activity of this fermentation product in the gastrointestinal tract. Other examples of *Candida*-bacterial antagonism via fatty acid metabolites have been reported. *Pseudomonas aeruginosa* can produce 15-hydroxyeicosatetraenoic acid (15HETE) from exogenous arachidonic acid and can grow on and kill hyphal *C. albicans* (18, 43). *Pseudomonas* is also found in low levels as part of the bacterial microflora and increases in number after antibiotic treatment (13, 20). The mechanism of *Pseudomonas*-*Candida* antagonism has been proposed to be multifactorial. Since we have demonstrated that 15HETE does not decrease or augment germination, it seems likely that this fatty acid metabolite does not play a role in the bacterial-fungal antagonism of these two microbes. Overall, these results indicate that fatty acid metabolic pathways can regulate germination of *C. albicans* and the local fatty acid environment can influence whether *C. albicans* exists as a commensal or pathogen.

**FIG. 5.** Effect of LAB culture supernatants on *C. albicans* morphogenesis. *Lactobacillus* spp. were grown in *Lactobacillus* MRS broth for (a) 2 h or (b) 24 h under microaerophilic conditions at 37°C. *Lactobacillus* culture supernatants (sups) from these two time points or carrier (MRS broth) was added to *C. albicans* diluted in 100% FBS at neutral pH. Germination was measured as described in Materials and Methods. The assay was performed in triplicate and repeated two times with similar results. *, *P* < 0.05 as determined by Student’s *t* test.

**FIG. 6.** Effect of SCFA on *C. albicans* morphogenesis. SCFA at various concentrations (Sigma Chemical Co., St. Louis, Mo.) or carrier (1× PBS) was added to *C. albicans* diluted in 100% FBS and adjusted to neutral pH. Germination was measured as described in the legend to Fig. 1. The assay was performed in triplicate and repeated two times with similar results. *, *P* < 0.05 as determined by Student’s *t* test.
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