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Lactic acid bacteria differentially regulate filamentation in two heritable cell types of the human fungal pathogen *Candida albicans*

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Abstract

Microorganisms rarely exist as single species in natural environments. The opportunistic fungal pathogen *Candida albicans* and lactic acid bacteria (LAB) are common members of the microbiota of several human niches such as the mouth, gut, and vagina. LAB are known to suppress filamentation, a key virulence feature of *C. albicans*, through the production of lactic acid and other metabolites. Here we report that *C. albicans* cells switch between two heritable cell types, white and opaque, to undergo filamentation to adapt to diversified environments. We show that acidic pH conditions caused by LAB and low temperatures support opaque cell filamentation, while neutral pH conditions and high temperatures promote white cell filamentation. The cAMP signaling pathway and the Rfg1 transcription factor play major roles in regulating the responses to these conditions. This cell type-specific response of *C. albicans* to different environmental conditions reflects its elaborate regulatory control of phenotypic plasticity.

Graphical abstract

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Complementary response to pH and temperature in *C. albicans*
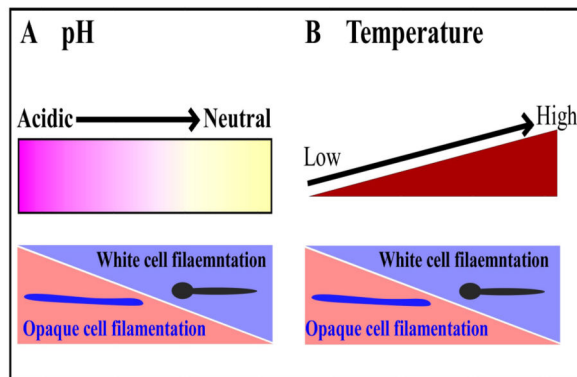
Introduction

Many natural environments, including specific niches within the human body, contain diverse populations of microbial species (Hibbing *et al.*, 2010, Morales & Hogan, 2010). Constant competition for space and nutritional resources in the same niche promotes the evolution of new biological traits in different species. The opportunistic fungal pathogen, *Candida albicans*, and lactic acid bacteria (LAB) co-inhabit a number of human niches, such as the mouth, gut, and female reproductive tract (Krasner *et al.*, 1956, Wargo & Hogan, 2006, Mason *et al.*, 2012, Wagner & Johnson, 2012). LAB are known to be protective species for the host, preventing overgrowth of *C. albicans* through the production of lactic acid and other metabolites (Lankaputhra & Shah, 1998, Boris & Barbes, 2000, Barrons & Tassone, 2008, Shirtliff *et al.*, 2009, Wagner & Johnson, 2012). In this study, we report that *C. albicans* uses a bet-hedging strategy to maintain its phenotypic diversity and to adapt to environmental changes in the presence of LAB.

C. albicans is typically harmless to healthy individuals, but it can cause serious infections in immunocompromised individuals or individuals who have received long-term, broad-spectrum antibiotic therapy (Berman, 2012, Brown *et al.*, 2012). A striking feature of *C. albicans* is its ability to change cellular morphologies in response to different environmental cues (Biswas *et al.*, 2007, Whiteway & Bachewich, 2007, Sudbery, 2011, Huang, 2012); it can grow as either a unicellular budding yeast form or as a multicellular filamentous form in response to a myriad of environmental stimuli (Sudbery, 2011). Relative to yeast cells, filamentous cells are better at adhering to and invading host tissues, while yeast cells are the primary commensal form and are better at dispersing throughout the body via the bloodstream, leading to disseminated infections (Koh *et al.*, 2008, Vautier *et al.*, 2015). The yeast-to-filament transition can be induced by a number of host-related environmental factors, including mammalian physiological temperature (37°C), serum, and neutral pH (Biswas *et al.*, 2007, Huang, 2012). Several pathways and a variety of transcription factors are involved in the regulation of yeast-filament transitions (Biswas *et al.*, 2007, Whiteway & Bachewich, 2007, Huang, 2012). White-opaque switching is another important morphological transition system in *C. albicans* (Slutsky *et al.*, 1987). White and opaque cells differ in cellular morphology and physiology, gene expression profiles, virulence, and

mating competency (Anderson & Soll, 1987, Slutsky *et al.*, 1987, Lan *et al.*, 2002, Miller & Johnson, 2002, Lohse & Johnson, 2009, Soll, 2009). Distinct from the yeast-filamentous growth transition, the switch between white and opaque cell types is bistable and epigenetically regulated (Anderson & Soll, 1987, Slutsky *et al.*, 1987, Huang *et al.*, 2006). White cells can be induced to form filaments in response to serum, neutral pH, and Lee's medium at 37°C, whereas opaque cells cannot undergo filamentation under these standard filament-inducing laboratory conditions (Anderson *et al.*, 1989, Guan *et al.*, 2013, Si *et al.*, 2013). In addition to white and opaque cell types, we recently discovered a novel heritable cell type, the gray phenotype, in *C. albicans*, *Candida dubliniensis*, and *Candida tropicalis* (Tao *et al.*, 2014, Yue *et al.*, 2016, Zhang *et al.*, 2016). Gray cells exhibit a set of distinct biological features from white and opaque cells, and the ability to switch between these three distinct cell states is likely important for adaptation of *Candida* species to the host environment.

A number of studies have demonstrated that interactions with bacteria can influence important biological processes in *C. albicans*, such as biofilm formation, morphogenesis, and pathogenesis (Shirtliff *et al.*, 2009, Harriott & Noverr, 2011, Fox *et al.*, 2014, Sztajer *et al.*, 2014, Trejo-Hernandez *et al.*, 2014). Both *C. albicans* and *Lactobacillus* species are common members of the microbiota of the human mouth, gut, and genital tract. *Lactobacillus* species are the predominant vaginal microorganisms in healthy women (Strus *et al.*, 2005, Ravel *et al.*, 2011) and are believed to play important roles in the control of *Candida* vaginitis in women and diaper dermatitis in infants. Organic acids (such as lactic acid) secreted by LAB decrease the pH level of the surrounding environment and thus repress filamentation of white cells of *C. albicans* (Scheppach, 1994, Lankaputhra & Shah, 1998, Noverr & Huffnagle, 2004, Shareck *et al.*, 2011). Other bacterial products, such as H_2O_2 and bacteriocin-like components can also induce cell death in *C. albicans* (Morales & Hogan, 2010, Parolin *et al.*, 2015). Given the dominant features of *Lactobacillus* species and their antagonistic activities on *C. albicans*, we wondered how these microorganisms could co-exist in the same niches (such as the vagina and skin). We discovered that when co-cultured with LAB or lactic acid, opaque cells of *C. albicans* undergo filamentation using in vitro assays. The cAMP signaling pathway and a number of transcription factors, including Rfg1, Nrg1, and Cup9, play critical roles in this regulation.

Results

Lactic acid bacteria induce filamentation in opaque cells, but not in white cells, of *C. albicans* in vitro

When co-cultured with cells of *Lactobacillus casei* (strain L7) on MRS medium plates at 25°C, opaque cells, but not white cells of *C. albicans*, underwent filamentation. However, both white and opaque cells of the single culture controls remained in the yeast form (Figs. S1, 1A and 1B). Three areas of the LAB-*C. albicans* co-culture colony were examined (central, close to edge, and edge areas, Fig. S1). In general, more filamentous cells were present in the colony edge area than the inner colony areas. Therefore, cells examined in subsequent experiments were picked from the edge area of a colony. Similarly, *L. casei* L7 induced opaque cell filamentation, but not white cell filamentation, in four genetically

independent *C. albicans* strains (Fig. S2A), suggesting that this response to LAB is a general feature of natural strains of *C. albicans*. We further tested the effect of 12 *Lactobacillus* strains, representing 11 different species, on the induction of filamentation in white and opaque cells of *C. albicans*. As shown in Fig. S3, all *Lactobacillus* strains induced filamentation in opaque cells, but not in white cells. However, cells of other interacting bacterial species, such as those of *Staphylococcus epidermidis* and *Escherichia coli* had no obvious effects on filamentation in white or opaque cells of *C. albicans*. These results suggest that the ability to induce opaque cell filamentation in *C. albicans* is a common and specific feature of *Lactobacillus* species. Since *L. casei* L7 showed the most potent inducing effect on opaque cell filamentation, it was used in all subsequent experiments.

Lactic acid bacteria induce opaque cell filamentation in *C. albicans* at 30°C

The physiological temperature of mammalian skin is about 30-32°C. We therefore performed filamentation assays at 30°C both in air and in 5% CO_2 (opaque cells are stable in the presence of high levels of CO_2). As shown in Fig. S4, only opaque cells underwent filamentation when co-cultured with cells of *L. casei* L7. Opaque cells of the single culture control and white cells co-cultured with *L. casei* L7 did not undergo filamentation.

Expression of opaque- and filament-enriched genes

Calcofluor White staining and cellular morphology assays demonstrated that LAB-induced opaque filaments are true hyphae (Fig. 2A), consisting of long hyphal tubes with no obvious constrictions. Three GFP reporter strains were constructed, in which the expression of GFP was placed under the control of the promoters of the opaque-enriched gene *OP4* and the filament-enriched genes *HWP1* and *ECE1*, respectively. Fluorescence microscopy assays demonstrated that both opaque-enriched (*OP4*) and filament-enriched (*HWP1* and *ECE1*) genes were expressed in LAB- or lactic acid-induced filamentous cells (Fig. 2B).To further verify the cell identities of the filamentous cells, we examined the relative expression levels of three opaque-enriched (*OP4*, *SAP1*, and *PHO89*) and three white-enriched (*EFG1*, *WH11*, and *HSP70*) genes by quantitative RT-PCR assays. As expected, these cell type-specific genes exhibited a similar expression pattern in the LAB-*C. albicans* co-cultures to that in opaque cells of the single culture (Fig. 2C)

Lactic acid produced by LAB regulates filamentation in opaque cells

All LAB strains tested were capable of inducing opaque cell filamentation in *C. albicans* (Fig. S3). The strain L7 was able to induce opaque cell filamentation in *C. albicans* when both species were grown separately, but adjacent to each other (direct interaction between cells of the two species is not required for this induction) (Fig. 1C), suggesting that secreted products of lactic acid bacterial cells have an effect on *C. albicans* opaque cell filamentation. A common metabolic and physiological feature of all LAB strains is the ability to produce lactic acid (L-lactic acid and/or D-lactic acid) and to acidify the surrounding environment. We suspected that lactic acid played a major role in the induction of opaque cell filamentation in *C. albicans*. As shown in Fig. 3, the addition of L-lactic acid or D-lactic acid to Lee's glucose medium (a standard laboratory medium for white-opaque switching assays (Lee *et al.*, 1975, Huang *et al.*, 2010)) induced filamentation in opaque cells, but not in white cells of *C. albicans*. The addition of L-lactic acid or D-lactic acid to the medium decreased

the pH level to about 4.0. To determine if this shift in pH was responsible for the observed opaque cell filamentation, we then adjusted the pH of the medium to 7.0 with sodium hydroxide (NaOH) and found that neither opaque cells nor white cells could undergo filamentation (Fig. 3C), suggesting that lactic acid-induced opaque cell filamentation is dependent on acidic pH conditions. Moreover, the addition of L-lactic acid or D-lactic acid to Spider and SD media also induced opaque cell filamentation (data not shown). However, we note that supernatants of LAB did not induce opaque cell filamentation perhaps due to the rapid pH change and consumption of lactic acid by *C. albicans* (data not shown). These results demonstrate that lactic acid and its pH lowering effect are important determinants of opaque cell filamentation in *C. albicans*.

Since lactic acid bacteria can also produce other organic acids, such as acetic acid, formic acid, and propanoic acid, we performed filamentation assays to test whether these organic acids can also induce filamentous growth in opaque cells of *C. albicans*. Only propanoic acid had a minor effect on the induction of opaque cell filamentation (data not shown). Since propanoic acid is not a major product of lactic acid bacteria, our study is focused on the role of lactic acid in the induction of opaque cell filamentation.

Acidic pH alone does not induce opaque cell filamentation in *C. albicans*

Since pH conditions are important for the lactic acid-induced opaque cell filamentation in *C. albicans*, we next performed filamentation assays on Lee's glucose media adjusted to different pH levels at 25°C. Consistent with our previous observation (Guan *et al.*, 2013), neither white cells nor opaque cells underwent obvious filamentation under all pH conditions tested, suggesting that both the acidic pH environment and the lactate ion play important roles in lactic acid-induced opaque cell filamentation in *C. albicans*.

Distinct responses of white and opaque cells to pH and temperature conditions

LAB produce organic acids and create an acidic environment, which represses white cell filamentation in *C. albicans* (Ramon *et al.*, 1999, O'Hanlon *et al.*, 2013, Cornet & Gaillardin, 2014). Consistently, we found that on Lee's glucose medium white cells rarely underwent filamentation at pH4.0 to 6.0. White cells showed gradually increased filamentation ability at pH levels of 6.5 to 8.0 (Fig. 4A; we note that the growth of *C. albicans* cells can also slightly acidify the medium, therefore, the actual pH of the medium over time may be lower than that of the original medium). However, opaque cells underwent filamentation at pH 4.0 to 5.0, but not at pH levels great than 6.0. In general, the more acidic the environment, the greater the number of opaque cell filaments were observed (Fig. 4A). These results suggest that white and opaque cells show a complementary response feature to pH conditions.

We further found that high temperature conditions favored white cell filamentation and repressed opaque cell filamentation. As shown in Fig. 4B, white cells showed a gradually increased ability to filament when incubated under increased temperature conditions (at 27°C, 30°C, 32°C, 34°C, and 37°C). However, opaque cells only underwent filamentation at relatively low temperatures (25°C and 27°C), consistent with a previous study (Si *et al.*, 2013) showing that high temperature conditions repress opaque cell filamentation. Together,

these results suggest that white and opaque cells show a complementary response feature to different environmental temperatures.

Role of the pH sensing pathway (Phr1, Phr2, and Rim101) in the regulation of opaque cell filamentation in *C. albicans*

The conserved Rim101 pathway plays an important role in the control of morphological transitions in response to environmental pH changes in *C. albicans* (Davis *et al.*, 2000, Davis, 2003). We next examined whether this pathway regulates lactic acid-induced opaque cell filamentation in *C. albicans*. White cells served as the control. Under all three culture conditions (on YPD, YPD+50mM L-lactic acid, and YPD+50mM D-lactic acid media, at 25 °C), white cells of both the WT, *phr1/phr1*, *phr2/phr2*, and *rim101/rim101* mutants did not undergo filamentation (Fig. S5). *PHR2* is expressed at an ambient pH below 5.5 and is required for normal growth at this pH (Fonzi, 1999). As expected, deletion of *RIM101* or *PHR1* attenuated the ability of opaque cells to undergo filamentation in *C. albicans* in the presence of L-lactic acid or D-lactic acid. Deletion of *PHR2* led to growth defects in the medium containing 50 mM L-lactic acid (or D-lactic acid) (Fig. S5). These results indicate that the Rim101-mediated pH sensing pathway plays a role in the regulation of lactic acid-induced opaque cell filamentation in *C. albicans*.

Role of the cAMP signaling pathway in the regulation of LAB-induced opaque cell filamentation in *C. albicans*

The cAMP signaling pathway is required for both white cell and opaque cell filamentation in response to different environmental cues (Leberer *et al.*, 2001, Rocha *et al.*, 2001, Guan *et al.*, 2013, Si *et al.*, 2013). As shown in Figs. 5 and S6, deletion of *RAS1*, encoding a small GTPase in *C. albicans*, dramatically attenuated LAB- or L-lactic acid-induced filamentation in opaque cells. Deletion of *CYR1*, the single gene encoding adenylyl cyclase in *C. albicans*, almost completely blocked opaque cell filamentation under both culture conditions. Tpk1 and Tpk2 are two isoforms of the cAMP-dependent protein kinase catalytic subunit (Bockmuhl *et al.*, 2001). Deletion of *TPK1* or *TPK2* did not completely block LAB-induced or L-lactic acid-induced filamentation, but did attenuate filamentation in opaque cells. These results indicate that the cAMP signaling pathway plays a critical role in the regulation of LAB or lactic acid-induced opaque cell filamentation.

Role of Efg1, Flo8, and Hgc1 in LAB-induced opaque cell filamentation

The transcription factors Efg1 and Flo8 function downstream of the cAMP signaling pathway and are required for filamentation in both white cells and opaque cells in response to different environmental cues (Lo *et al.*, 1997, Cao *et al.*, 2006, Guan *et al.*, 2013, Si *et al.*, 2013). We next examined whether they played similar roles in LAB- or lactic acid-induced opaque cell filamentation in *C. albicans*. Deletion of *EFG1* attenuated, but did not block, opaque cell filamentation induced by LAB or L-lactic acid. However, deletion of *FLO8* completely blocked opaque cell filamentation under both culture conditions. Since deletion of *FLO8* promotes opaque-to-white switching in *C. albicans*, we introduced an *ACT1* promoter-controlled *WOR1* gene into the *flo8/flo8* mutant to stabilize the opaque phenotype. The cyclin-related protein Hgc1 is regulated by Efg1 and Flo8 and is essential for filamentous growth in *C. albicans* (Zheng *et al.*, 2004, Cao *et al.*, 2006). Deletion of *HGC1*

locked opaque cells in the yeast form in the presence of either LAB or L-lactic acid (Figs. 5 and S6).

Role of Rfg1 in LAB- and lactic acid-induced opaque cell filamentation

The Rfg1 transcription factor plays a repressing role in the regulation of filamentous growth and the expression of filament-enriched genes in white cells, but has been reported as a positive regulator in the regulation of environmental cues-induced opaque cell filamentation (Si *et al.*, 2013). Since opaque cells of the *rfg1/rfg1* mutant were not stable, we introduced an *ACT1* promoter-controlled *WOR1* gene into the mutant to stabilize its opaque phenotype. As shown in Fig. 6, both LAB and L-lactic acid could induce filamentation in opaque cells of the *rfg1/rfg1* mutant. In the presence of 50 mM L-lactic acid, about 8% of opaque colonies were highly wrinkled (all the colonies of the WT+ACT1p-WOR1 control showed a rough or slightly wrinkled surface). Wrinkled colonies contained more filamentous cells than rough colonies. These results are inconsistent with the study of Si et al. (2013) (Si *et al.*, 2013), which demonstrated that Rfg1 is required for opaque cell formation. To clarify this discrepancy, we requested these strains from the authors (Si *et al.*, 2013) and found that opaque cells of their *rfg1/rfg1* mutants also showed an increased ability to filament (data not shown); we note that opaque cells of the strains from the Si *et al.* paper were not as stable as opaque cells of our strains, likely due to differences in the design of the *ACT1* promoter-controlled *WOR1* construct. These results suggest that as previously reported in white cells (Kadosh & Johnson, 2001), Rfg1 plays a repressing role in the regulation of LAB- or lactic acid-induced filamentation in opaque cells.

Role of Bcr1, Nrg1, and Cup9 in LAB- and lactic acid-induced opaque cell filamentation

The biofilm and opaque cell filamentation regulator Bcr1 represses opaque cell filamentation and hypoxia-induced white cell filamentation in *C. albicans* (Nobile & Mitchell, 2005, Fanning *et al.*, 2012, Guan *et al.*, 2013). As expected, the *bcr1/bcr1* mutant underwent opaque cell filamentation under all culture conditions (Figs. 5 and S6). Nrg1 and Cup9 are conserved general transcriptional repressors in *C. albicans* (Braun *et al.*, 2001, Guan *et al.*, 2013). On YPD medium, both white and opaque cells of the *nrg1/nrg1* mutant underwent extensive filamentation (Fig. S7). In the presence of 50 mM L-lactic acid, white and opaque cells formed colonies with distinct morphologies. Opaque cells underwent more extensive filamentation compared to white cells; opaque colonies of the *nrg1/nrg1* mutant were fuzzy and hard, and more invasive than white colonies. Deletion of *CUP9* had no obvious effect on white cell filamentation, but increased opaque cell filamentation induced by lactic acid. These results suggest that Nrg1 and Cup9 play critical roles in the regulation of lactic acid-induced opaque cell filamentation.

Discussion

Phenotypic plasticity is a striking feature of *C. albicans*. Different morphologies play distinct roles in the commensal and pathogenic lifecycles of this fungus. In the current study, we report that *C. albicans* integrates the white-opaque switching system into the regulation of filamentation in response to pH (induced by its co-habitant *Lactobacillus* species) and temperature changes. We sugest that the distinct responses to pH and temperature

fluctuations in white and opaque cells represent a bet-hedging strategy, which promotes filamentous growth in a portion of *C. albicans* cells under different environmental conditions (Fig. 4).

The human body harbors numerous microorganisms. In healthy women, *Lactobacillus* species dominate the vaginal microbiota (Ravel *et al.*, 2011, Parolin *et al.*, 2015). It is well documented that LAB have an antagonistic effect on *C. albicans* cells by producing toxic organic acids, H_2O_2, and antifungal metabolites (Boris & Barbes, 2000, Barrons & Tassone, 2008, Parolin *et al.*, 2015). Moreover, LAB cells compete with *C. albicans* cells for adhesion sites and thus defend the host against colonization by this opportunistic fungal pathogen (Wargo & Hogan, 2006, Morales & Hogan, 2010). We found that LAB acidify the surrounding environment by producing organic acids (primarily lactic acid), which suppresses white cell filamentation, but exclusively promotes filamentation in opaque cells of *C. albicans* (Figs. 1 and S3). As depicted in Fig. 4, the pH and temperature responses in white and opaque cells exhibit a distinct and complementary feature. Neutral-to-alkaline pH promotes filamentous growth in white cells, while acidic pH induces filamentation in opaque cells; low temperatures (<30°C) favor opaque cell filamentation, while high temperatures (>30°C) promote white cell filamentation. This is clinically relevant because the physiological temperatures of the skin, out region of the vagina, and babies' diapers, for example, could be lower than 30°C, thereby promoting filamentation of specific *C. albicans* cell types over others. In general, the ability to form filaments is an important virulence factor of *C. albicans*, which can increase adherence and promote its colonization in these niches. Therefore, this cell type-specific response to LAB and temperature changes likely represents an adaptive behavior of *C. albicans* in specific physiological environments. The interactions between *C. albicans* and *Lactobacillus* species described here were primarily performed in vitro. Further investigations are needed to determine the effects of *Lactobacillus* species on filamentation of white and opaque cells in *C. albicans* in natural niches, such as the human host.

Distinct programs are involved in the regulation of filamentation in white and opaque cells of *C. albicans* (Guan *et al.*, 2013, Si *et al.*, 2013). A number of natural or host-related environmental cues induce filamentation in white cells, such as serum, CO_2, and oxidative stresses (Biswas *et al.*, 2007, Huang, 2012). Several laboratory culture media (such as SOR medium, which contains 1 M sorbitol, and MIN medium, which lacks amino acids) have recently been reported to favor filamentation in opaque cells (Si *et al.*, 2013). However, these artificial conditions are unlikely to represent typical host environments. In this study, we discovered that acidic pH conditions, which are attributed predominantly to the production of lactic acid by LAB, represent a natural host environment that promotes opaque cell filamentation.

The conserved Rim101 pathway controls the pH response in *C. albicans* (Davis *et al.*, 2000, Davis, 2003). *RIM101* itself is a pH-response gene, which is induced at alkaline pH and is required for pH-regulated filamentation in white cells (Davis *et al.*, 2000). *PHR1* and *PHR2*, encoding two pH sensors in *C. albicans*, are transcriptionally regulated by Rim101 (De Bernardis *et al.*, 1998, Fonzi, 1999, Davis *et al.*, 2000). *PHR1* is expressed when the ambient pH is 5.5 or higher, while *PHR2* is expressed at a pH below 5.5. *PHR2* is essential for cell

growth at pH 4.0 or in the presence of 50 mM lactic acid. Rim101 and Phr1 are not essential for lactic acid-induced filamentation. However, deletion of *RIM101* or *PHR1* attenuated filamentation in opaque cells (Fig. S5). These results suggest that the Rim101-mediated pH sensing pathway does not play a major role in the regulation of lactic acid-induced opaque cell filamentation. Consistent with this idea, decreased pH alone in Lee's medium does not induce opaque cell filamentation (Guan *et al.*, 2013). Moreover, Cottier *et al.* (2015) recently demonstrated that *C. albicans* cells treated with HCl or lactic acid show distinct transcriptional profiles (Cottier *et al.*, 2015). Although the pH levels of the media were the same in the two conditions, there could be a synergistic effect of pH and lactate ions on the induction of opaque cell filamentation.

The cAMP signaling pathway plays a central role in both white- and opaque-cell filamentation (Guan *et al.*, 2013, Si *et al.*, 2013). Deletion of either *TPK1* or *TPK2* did not completely block opaque cell filamentation, possibly due to their redundant roles in the regulation of downstream transcription factors. As expected, deletion of *CYR1*, *FLO8*, or *HGC1* completely blocked opaque cell filamentation induced by LAB or lactic acid. Efg1, a downstream regulator of the cAMP signaling pathway, is known to play an important role in opaque cell filamentation induced by SOR or MIN medium (Si *et al.*, 2013). However, in our study, we found that Efg1 is not required for opaque cell filamentation induced by either LAB or lactic acid, although the *efg1/efg1* mutant exhibited notably attenuated filamentation (Figs. 5 and S6). Efg1 plays a critical role in the regulation of filamentation, white-opaque switching, and biofilm formation (Lo *et al.*, 1997, Sonneborn *et al.*, 1999, Zordan *et al.*, 2007, Nobile *et al.*, 2012). Given its general roles on transcriptional regulation of many developmental processes, the effect of inactivating Efg1 on attenuating filamentation is not surprising. Consistent with our previous study (Guan *et al.*, 2013), deletion of *EFG1* in opaque cells did not completely block filamentation. In fact, deletion of *EFG1* was found to promote filamentation under embedded culture conditions (Cao *et al.*, 2006). These studies suggest that Efg1 is not the master regulator of opaque cell filamentation and is not essential for white cell filamentation under certain conditions. Deletion of *RFG1* caused a portion of the colonies to undergo robust filamentous growth (Fig. 6), suggesting that the Rfg1 transcription factor plays a repressing role in lactic acid-induced opaque cell filamentation. This regulatory mechanism is similar to that seen in white cells (Kadosh & Johnson, 2001, Kadosh & Johnson, 2005). The transcriptional repressors Cup9 and Nrg1 are also involved in the regulation of opaque cell filamentation induced by LAB or lactic acid (Fig. S7). We previously demonstrated that the master regulator of opaque cell filamentation, Bcr1, binds to the promoter regions of *EFG1*, *CUP9* and *NRG1* (Guan *et al.*, 2013). These results, together with our findings in this study, suggest that multiple pathways are involved in the regulation of LAB-induced opaque cell filamentation in *C. albicans* (Fig. 7).

Conclusion

To survive and thrive in natural environments, microorganisms use different strategies to acquire nutrient resources and battle for space. To avoid being overpopulated by neighboring microbial members, such as *Lactobacillus* species, *C. albicans* adopts different cellular morphologies and undergoes cell type-specific filamentation by initiating distinct regulatory

programs. Our results suggest that *C. albicans* has evolved different mechanisms in different cell types to respond to environmental changes.

Experimental procedures

Strains, plasmids, and culture conditions

The strains and primers used in this study are listed in Tables S1 and S2, respectively. The detailed methods for construction of strains and plasmids are described in the supplementary methods. Lactic acid bacteria (LAB) were routinely grown in liquid MRS medium or on MRS agar plates (Rogosa & Sharpe, 1960). MRS medium composition (per liter): 10 g beef extract, 5 g yeast extract, 10 g casein peptone, 20 g glucose, 5 g sodium acetate, 1 ml tween 80, 2 g ammonium citrate dibasic, 2 g K_2HPO_4, 0.58 g $MgSO_4 \cdot 7H_2O$, 0.25 g $MnSO_4 \cdot H_2O$, 15 g agar. *C. albicans* strains were routinely grown on YPD agar (10 g yeast extract, 20 g peptone, 20 g dextrose, and 20 g agar for 1 L) or modified Lee's medium agar (Lee *et al.*, 1975, Xie *et al.*, 2013). MRS medium was used for mixed cultures of *C. albicans* and LAB; both *C. albicans* and LAB can grow well in this medium (Kohler *et al.*, 2012). The pH of the medium was adjusted with 37% hydrogen chloride (HCl) or 1 M sodium hydroxide (NaOH). Opaque cell filamentation on SOR medium was performed as described previously (Si *et al.*, 2013). All filamentation assays were performed in vitro at least three times. A set of representative data are presented in each figure. For filamentation assays performed on solid agar plates, cells examined were picked from the edge area of a colony (except for Fig. S1). The degree of filamentation is indicated by the number of "+" symbols or percentages of filamentous cells. To examine the cell identities of *C. albicans* cells in the co-cultures, cells were resuspended in ddH2O, diluted, replated onto Lee's glucose plates and incubated at 25°C for five days. Ratios of white and opaque colonies were then examined. To measure the pH of LAB-*C. albicans* co-cultured solid media, the nutrient agar (containing 0.5% agar) was first frozen at −80°C for one day and then thawed out. The agar was transferred to a centrifuge tube and spun at 5, 000 RPM. The supernatant was collected and used for pH determination.

Microscopy

Cells collected from nutrient agar plates were used for morphological analysis. Differential interference contrast (DIC) optics were used for standard cellular morphological examination. To discriminate hyphae and pseudohyphae, cells were stained with Calcofluor White and imaged by fluorescent microscopy as previously described (Guan *et al.*, 2013). To detect the expression of GFP in reporter strains (under the control of the promoters of filament-enriched or opaque-enriched genes), cells were collected from nutrient agar plates and imaged by fluorescence microscopy.

Quantitative real-time PCR (Q-RT-PCR) assay

Cells of the single cultures or LAB-*C. albicans* co-cultures were grown on MRS medium at 25°C for three days. Total RNA was extracted using GeneJET RNA Purification kits according to the manufacturer's instructions. Quantitative RT-PCR assays were performed as previous described (Xie *et al.*, 2013, Zhang *et al.*, 2016). The signal from each experimental sample was normalized to expression of the *ACT1* gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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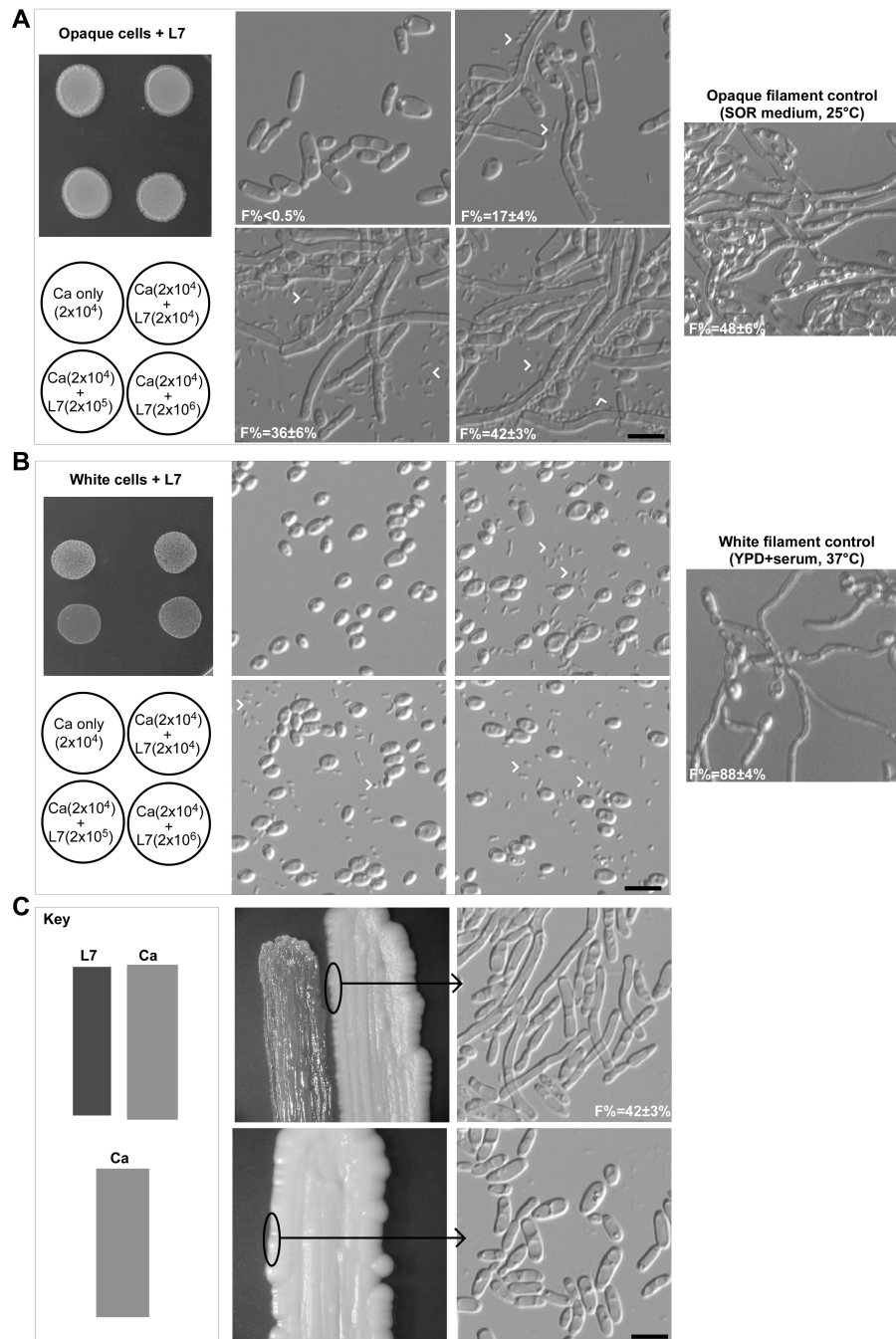
Fig. 1. Lactic acid species L7 (*Lactobacillus casei*) induces filamentation in opaque cells, but not in white cells, of *C. albicans*

Opaque (A) or white (B) cells (2×10^4) of *C. albicans* were mixed with different amounts of bacterial cells (in 20 μL ddH$_2$O) and incubated on MRS medium plates at 25°C for three days. Opaque filament control, cells incubated on SOR medium at 25°C for five days; White filament control, cells incubated on YPD + serum medium at 37°C for three days. (C) Secreted metabolites of lactic acid species L7 regulate opaque cell filamentation. Cells of *C. albicans* and L7 were patched on MRS medium plates and incubated on MRS medium plates at 25°C for three days. The *C. albicans* single culture patch served as the control. *C. albicans*

strain GH1349, a WO-1 background strain, was used. Scale bar, 10 μm. In this figure and all the subsequent figures, filamentation assays were repeated at least three times and a set of representative images are presented. In the co-cultures, over 95% of *C. albicans* cells maintained their original phenotypes (tested by replating assays). F%, percentage of filamentous cells. No filamentous cells in (B) were observed (F%<0.1%).
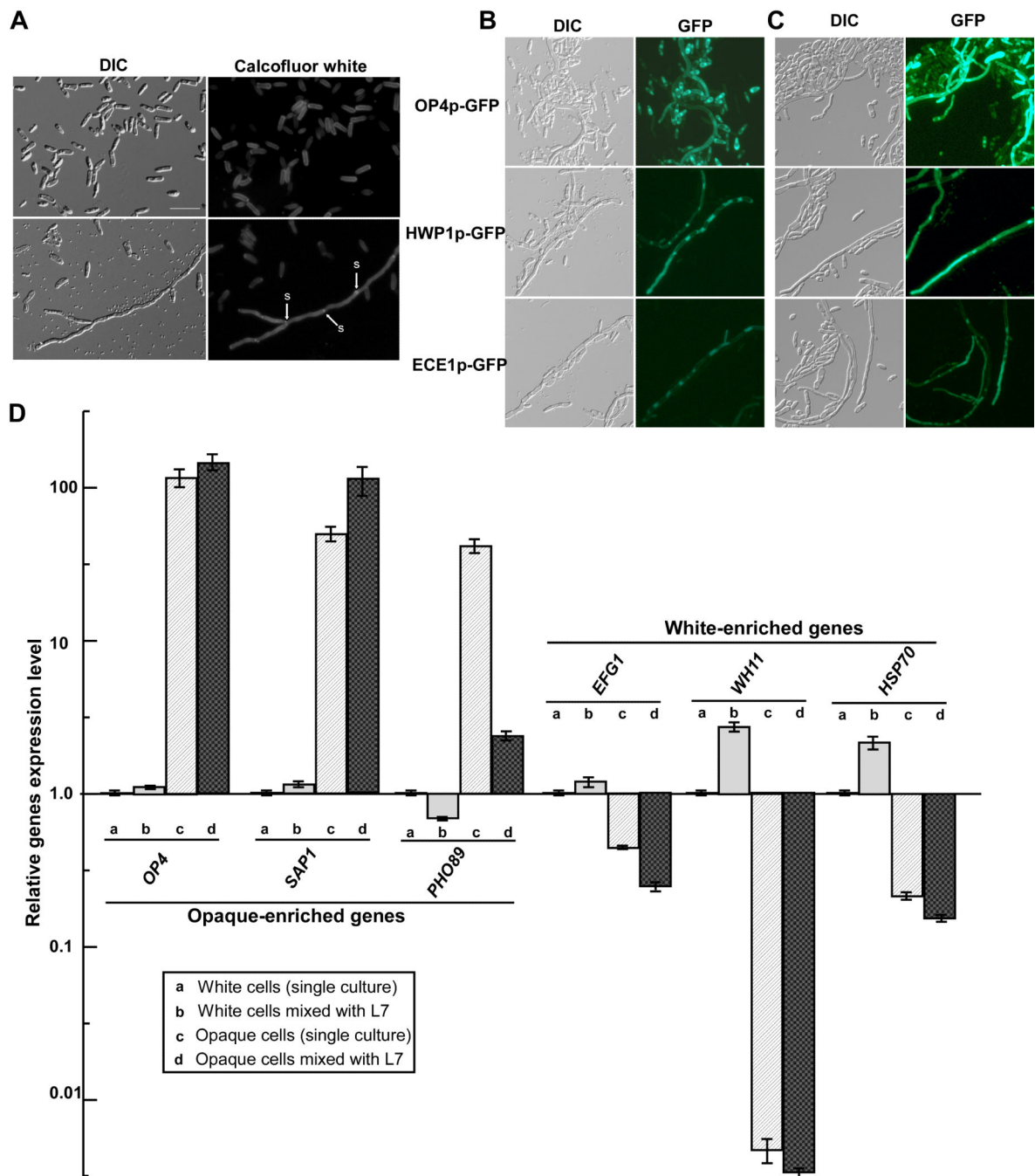
Fig. 2. Expression of filamentous- and opaque-enriched genes in *C. albicans* cells co-cultured with lactic acid bacterium L7 or treated with L-lactic acid

C. albicans strain GH1349 was used. (A) Calcofluor White staining assay. Opaque cells (2×10^4) of *C. albicans* and cells of L7 (2×10^6) were co-cultured on MRS medium plates at 25°C for three days. Single culture of opaque cells of *C. albicans* served as the control. Cells were stained with Calcofluor White and imaged. (B) Expression of filamentous (*HWP1* and *ECE1*)- and opaque (*OP4*)-enriched genes in *C. albicans* cells co-cultured with lactic acid bacterium L7. Opaque cells (2×10^4) of *C. albicans* and cells of L7 (2×10^6) were co-cultured on MRS medium plates at 25°C for three days. (C) Expression of

filamentous(*HWP1* and *ECE1*)- and opaque(*OP4*)-specific genes in *C. albicans* cells grown on YPD medium containing 50 mM L-lactic acid. Cells were cultured at 25°C for five days. (D) Relative expression levels of white and opaque cell type-enriched genes in single culture or LAB-*C. albicans* co-cultures (on MRS medium for three days at 25°C). The value of the expression level of each gene in white cells of the single culture was set as "1".
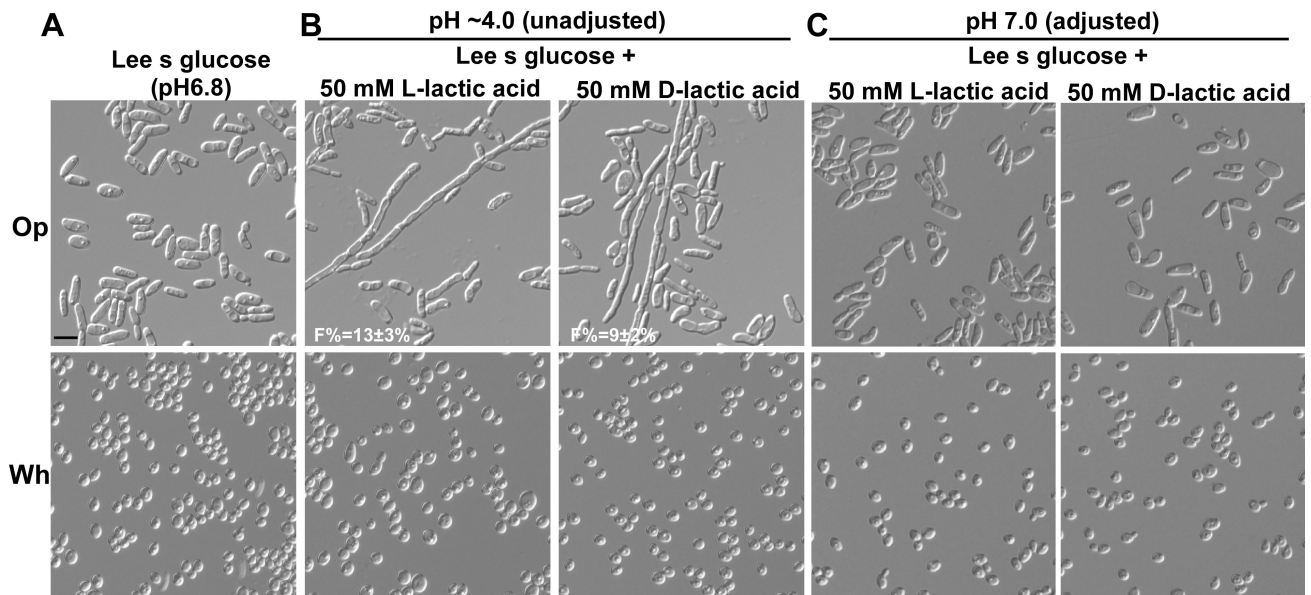
Fig. 3. Lactic acid induces filamentation in opaque cells of *C. albicans*
C. albicans strain GH1349, a WO-1 background strain, was used. Opaque or white cells were plated onto different medium plates and incubated at 25°C for five days. Scale bar, 10 μm. (A) Lee's glucose medium (pH6.8) served as the control. (B) Lee's glucose plates containing 50 mM L-lactic acid or 50 mM D-lactic acid (pH ~4.0, unadjusted). (C) Lee's glucose plates containing 50 mM L-lactic acid or 50 mM D-lactic acid (pH ~7.0, adjusted with NaOH). F%, percentage of filamentous cells. If no filamentous cells were observed (F%<0.1%), the percentages are not shown.
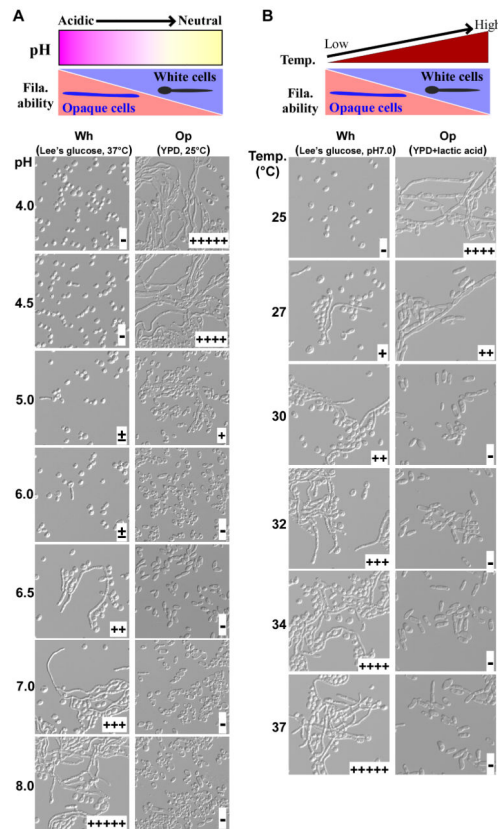
Fig. 4. White and opaque cells exhibit a complementary feature in response to different pH and temperature conditions

Fila. ability, filamentation ability; Temp., temperature; wh, white cells; op, opaque cells. "+", indicates the degree of filamentation; "−", no obvious filamentous cells observed. (A) pH differentially regulates white and opaque cell filamentation. Opaque cells (SN152, /α) were plated onto modified YPD medium plates (adjusted to pH 4.0 to pH 8.0 as indicated) and incubated at 25°C for five days. White cells (SN152, /α) were plated onto Lee's glucose medium plates (adjusted to pH 4.0 to pH 8.0 as indicated) and incubated at 37°C for five days. (B) Temperature differentially regulates white and opaque cell filamentation. White and opaque cells incubated at different temperatures (25°C to 37°C) as indicated for five days. Lee's glucose medium (pH 6.8) was used for the growth of white cells (SN152, /α), while modified YPD medium (containing 50 mM L-lactic acid) was used for the growth of opaque cells (SN152, /α +pACT1-WOR1). The WOR1-ectopic expression strain was used for opaque cell filamentation because opaque cells of the WT strain are not stable at high temperatures (>27°C). Lee's and YPD media were used for white and opaque cell growth, respectively, because the former medium favors white cell filamentation, while the latter medium favors opaque cell filamentation. Final pH of the media: Lee's media maintained their original pHs; compared to the original pHs, final pHs of YPD medium were lowered ~0.8 unit after co-culture with LAB.
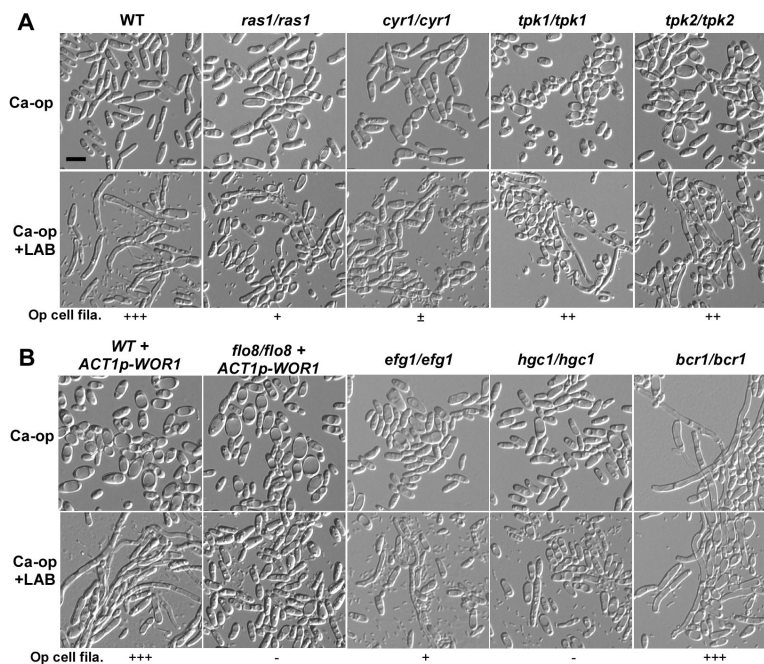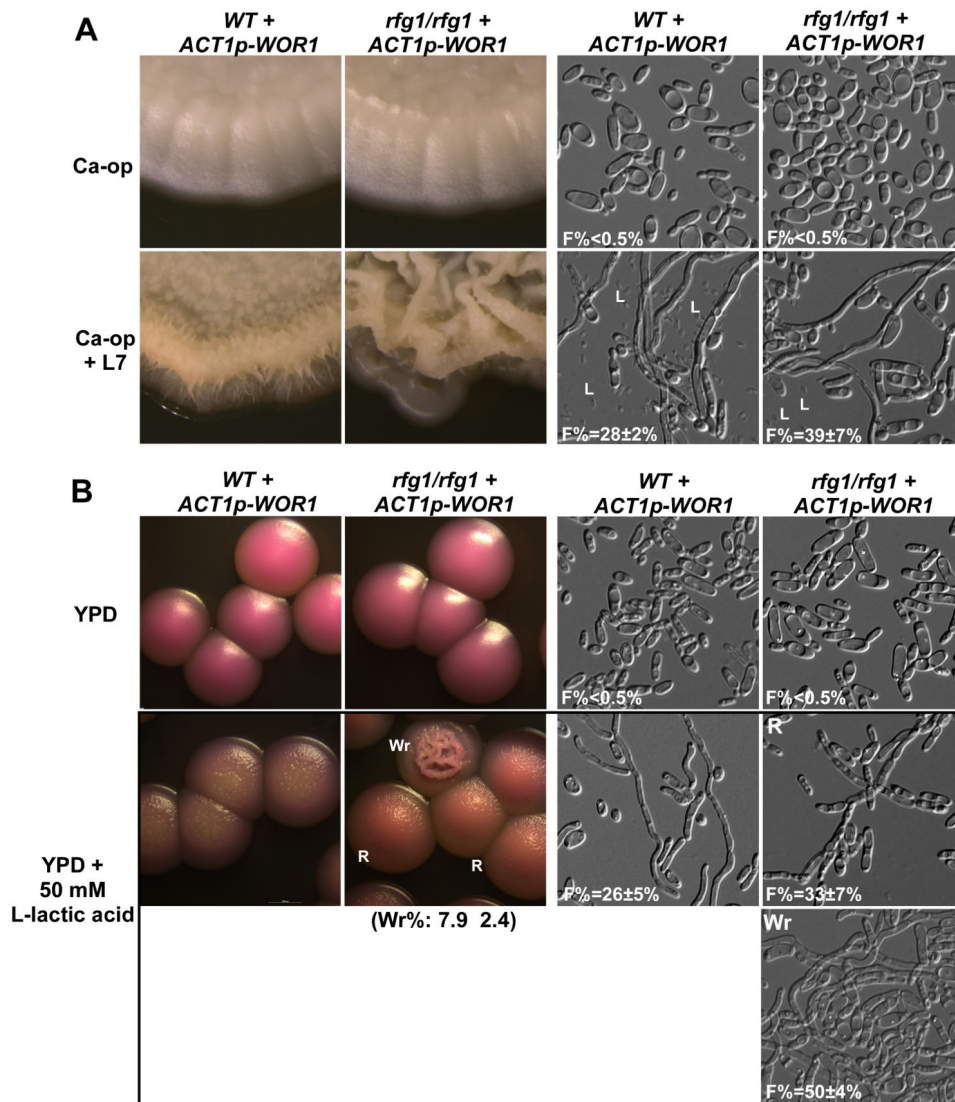
Fig. 5. Role of the cAMP signaling pathway, Hgc1, and Bcr1 in the regulation of LAB-induced opaque cell filamentation in *C. albicans*

Opaque cells (2×10^4) of *C. albicans* were mixed with bacterial cells of L7 and incubated on MRS medium plates at 25°C for three days. Ca-op, opaque cells of *C. albicans*; Ca-op +LAB, opaque cells of *C. albicans* mixed with bacterial cells of L7. (A) Cellular morphologies of the WT and mutants of the cAMP signaling pathway. WT, GH1349, which is a WO-1 background strain (same as the *ras1/ras1*, *cyr1/cyr1*, *tpk1/tpk1*, *tpk2/tpk2*, *hgc1/hgc1*, *bcr1/bcr1* and *efg1/efg1* mutants). (B) The WT (WT+ACT1p-WOR1) and mutants of *FLO8*, *EFG1*, *HGC1*, and *BCR1*. Scale bar, 10 μm. WT, GH1013, a SC5314 background strain (same as the *flo8/flo8* mutant). Cell identities after three days of co-culture (tested by replating assays): over 95% cells of the WT, *cyr1/cyr1*, *tpk2/tpk2*, *efg1/efg1*, and *hgc1/hgc1* mutants and about 70% cells of the *tpk1/tpk1* and *ras1/ras1* mutants maintained the opaque phenotype; Strains WT+pACT-WOR1 and *flo8/flo8*+pACT-WOR1 were locked in the opaque cell type. Two additional prototrophic controls of the WTs (GH1349 + ARG4 and SN250α + ARG4) are presented in Fig. S2B.

Fig. 6. Role of the transcription factor Rfg1 in the regulation of LAB or lactic acid-induced opaque cell filamentation

(A) Opaque cells (2×10^4) of *C. albicans* were mixed with 2×10^6 bacterial cells and incubated on MRS medium plates at 25°C for three days. Single culture of opaque cells served as the control. Ca-op, opaque cells of *C. albicans*; Ca-op+LAB, opaque cells of *C. albicans* mixed with bacterial cells of L7. (B) Opaque cells of *C. albicans* were incubated on YPD or YPD+L-lactic acid medium at 25°C for five days. Both wrinkled and rough colonies grown on lactic acid plates contained filamentous cells. For the *rfg1/rfg1* mutant, cells of a rough colony were imaged. The WT *C. albicans* strain SN250a served as the control. Wr%, percentage of wrinkled colonies. Wr, wrinkled colonies; R, rough colonies. Scale bar, 10 μm.
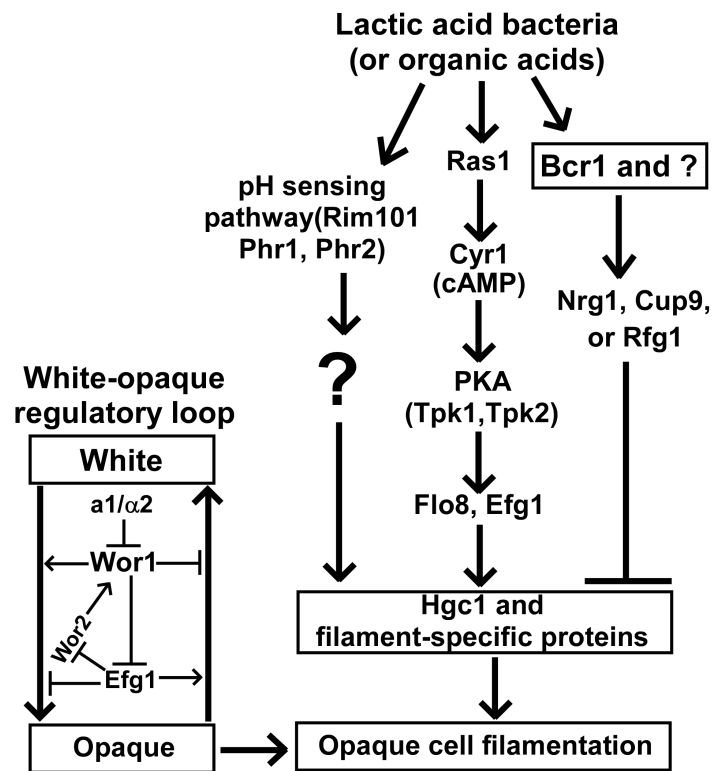
Fig. 7. Pathways of LAB-regulated opaque cell filamentation in *C. albicans*
Regulatory pathways of LAB- or lactic acid-induced opaque cell filamentation. The cAMP signaling pathway plays a positive role, while Bcr1, Nrg1, Cup9, and Rfg1 play a repressing role in the regulation of opaque cell filamentaion. White-opaque switching regulatory loop adapted from the reference (Zordan *et al.*, 2007).