The Rim101p/PacC Pathway and Alkaline pH Regulate Pattern Formation in Yeast Colonies

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Manuscript received November 20, 2009
Accepted for publication December 23, 2009

ABSTRACT

Multicellular organisms utilize cell-to-cell signals to build patterns of cell types within embryos, but the ability of fungi to form organized communities has been largely unexplored. Here we report that colonies of the yeast *Saccharomyces cerevisiae* formed sharply divided layers of sporulating and nonsporulating cells. Sporulation initiated in the colony's interior, and this region expanded upward as the colony matured. Two key activators of sporulation, *IME1* and *IME2*, were initially transcribed in overlapping regions of the colony, and this overlap corresponded to the initial sporulation region. The development of colony sporulation patterns depended on cell-to-cell signals, as demonstrated by chimeric colonies, which contain a mixture of two strains. One such signal is alkaline pH, mediated through the Rim101p/PacC pathway. Meiotic-arrest mutants that increased alkali production stimulated expression of an early meiotic gene in neighboring cells, whereas a mutant that decreased alkali production (*cit1Δ*) decreased this expression. Addition of alkali to colonies accelerated the expansion of the interior region of sporulation, whereas inactivation of the Rim101p pathway inhibited this expansion. Thus, the Rim101 pathway mediates colony patterning by responding to cell-to-cell pH signals. Cell-to-cell signals coupled with nutrient gradients may allow efficient spore formation and spore dispersal in natural environments.

IN metazoans, the hallmark of cell patterning is the establishment of sharp boundaries between cells of different fates. These boundaries are established and maintained by cell-to-cell signals. Even simple organisms can differentiate into several cell types, and these cell types can be organized into patterns within a larger community. For example, different cell types are organized within biofilms of the pathogenic yeast, *Candida albicans* (BAILLIE and DOUGLAS 1999), and within the fruiting body of the slime mold, *Dictyostelium discoideum* (KIMMEL and FIRTEL 2004; STRMECKI *et al.* 2005). However, in most cases communities of microorganisms have not been shown to contain sharp boundaries between cell types, and the role if any of cell-to-cell signals in organizing cell types within fungal communities is not known.

The yeast *Saccharomyces cerevisiae* may prove a genetically tractable model for fungal cell patterning (reviewed in PALKOVA and VACHOVA 2006). For example, periodic ammonia production during colony maturation can subdivide colonies into regions of cell growth and cell death (VACHOVA and PALKOVA 2005), colonies can form a skin-like cell layer on their surface (VACHOVA

et al. 2009), and *S. cerevisiae* can form elaborate biofilm-like structures on agar surfaces (REYNOLDS and FINK 2001). Finally, yeast colonies preferentially contain sporulated cells at their edge (PURNAPATRE and HONIGBERG 2002). Localization of sporulated cells (termed asci) to the edge of colonies may be related to variations in nutrient microenvironments in different regions of the colony. For example, because growth inhibits sporulation (COLOMINA *et al.* 1999; PURNAPATRE *et al.* 2002), regions of the colony that are more depleted for nutrients might sporulate more efficiently.

In addition to the inhibition of sporulation under growth conditions, sporulation is activated only after fermentable carbon sources such as glucose are fully metabolized to nonfermentable carbon sources such as ethanol (reviewed in HONIGBERG and PURNAPATRE 2003; KASSIR *et al.* 2003). Nonfermentable carbon sources stimulate sporulation directly, and metabolism of these carbon sources (via respiration) can also promote sporulation (JAMBHEKAR and AMON 2008). Respiration causes an increase in the pH of the media as a result of cells secreting bicarbonate, and this increased pH may further stimulate sporulation in cultures (OHKUNI *et al.* 1998). Activation of sporulation by alkaline pH might occur through the highly conserved Rim101/PacC pathway (PENALVA and ARST 2004), since this pathway activates transcription of many genes that

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.109.113480/DC1>.

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respond to alkaline pH, including genes required for sporulation (LAMB and MITCHELL 2003; ROTHFELS *et al.* 2005). However, a direct connection between alkaline pH and the Rim101 pathway in regulating sporulation has not been demonstrated. In the current study, we report on spatial patterns of sporulation that develop as colonies mature and the role of pH signals and the Rim101p pathway in regulating these patterns.

MATERIALS AND METHODS

Strains and media: Yeast strains (supporting information, Table S1) were constructed in the W303 background (LEE and HONIGBERG 1996) using standard methods (KAISER *et al.* 1994); all strains compared in this study contained identical auxotrophies. Construction of genomic *LacZ*-fusion alleles has been described (PURNAPATRE *et al.* 2002). To maximize sporulation, colonies were grown on YNA medium [2% potassium acetate (pH 7.0), 0.25% yeast extract, 2% agar, supplemented with the minimal amino acids necessary to balance auxotrophies]. For experiments with *cit1Δ* mutants, strains were grown on SPS2-0.3 medium, which contains 0.3% glucose, 2% acetate, 0.25% ammonium sulfate, and 0.09% yeast nitrogen base (no amino acids or ammonium sulfate).

For measurement of *LacZ* expression, 0.04 mg/ml of X-gal was added to the media after autoclaving. For colonies inoculated from 0.5-μl spots, we employed YNA-2 media [6% potassium acetate (pH 7.0), 0.5% yeast extract, 2% agar], which decreased the time required for color development. To detect increased pH in the colony environment, 20 mg/liter of phenol red was added to YNA-2 medium before autoclaving. To measure the effect of pH on sporulation, 100 mM Tris-HCl at the indicated pH was added to the medium after autoclaving. To change the pH of YNA medium after 3 days of growth, a 6-mm well was made in the center of the plate using a Pasteur pipette and then filled with 130 μl of 1 M NaHPO₄ buffer (pH 8.0). Colonies 2–4 cm from the well were analyzed for sporulation patterns. All other media are as described (ROSE *et al.* 1990).

Chimeric colonies: For colonies started from spot inoculations, here termed “spot colonies,” 1×10^5 cells were spotted on YNA-2 plates containing X-gal in a volume of 0.5 μl. For chimeric spot colonies, equal concentrations of each strain from overnight cultures were mixed, harvested, resuspended in H₂O, and spotted as above, except as noted. Plates were incubated for 6 days and photographed (Olympus C-60 camera), and the digital images were analyzed as described below. To determine the proportion of reporter cells in chimeric colonies, we suspended colonies in water, spread ~500 cells on YPD medium, and replica plated the resulting colonies to sporulation (SP3⁺) medium for 3 days before overlaying this medium with top agar containing X-gal and incubating for an additional 3 days (SEREBRIISKII *et al.* 2000).

Cryosections: Colonies were grown for various times on YNA medium, and then an agar block containing one colony was frozen in OCT (detailed protocol in File S1). Twenty-micrometer sections were examined by light microscopy and quantified as described below.

Embedded sections: To embed colonies for sectioning, we modified a previously described method (SCHERZ *et al.* 2001), with a major change being the substitution of Spurr's reagent as embedding medium, as described in detail in File S1. We determined the distribution of asci within a colony by superimposing nine equal-sized rectangles on the colony image and counting all cells in each rectangle (200–350 cells).

FIGURE 1.—Patterns of sporulation in wild-type colonies. Approximately 300 cells/plate were inoculated on YNA medium and grown for 8 days at 30°. Isolated colonies were sectioned and then examined by light microscopy. (A) Cross section of embedded colony. Bar, 0.4 mm. (B) Higher magnification of approximately the region indicated by a box in A. The region of sporulation is indicated by a bracket marked “S”, and the unsporulated region indicated by a bracket marked “U”. The arrow indicates representative tetrad asci (four spores) and the arrowhead indicates representative dyad asci (two spores). Bar, 25 μm.

Image acquisition, processing, and statistical analysis: All microscope images were captured using a Colorview II camera and AnalySIS software (SIS). Images in Figures 1, 2, and 4 were adjusted for brightness and contrast using Canvas 8. Quantification of *prIME-LacZ* expression in cryosections and spot colonies used Image J (National Institutes of Health) and a plug-in scripted for this study (color histogram). To achieve the greatest discrimination between blue and white regions in the images, we used the inverse of the signal in the red channel. For the cryosections, the red signal in each bin was subtracted from the bin with the highest red signal. For spot colonies, the signal was subtracted from the signal observed in a control (*LacZ*[−]) colony. All data is expressed as mean ± SEM.

RESULTS

Colonies contain a sharply delimited layer of sporulated cells: Because sporulation occurs preferentially at the edge of colonies (PURNAPATRE and HONIGBERG 2002), we investigated the pattern of sporulation within colonies. For this purpose, we embedded and sectioned colonies incubated for 8 days on YNA medium (Figure 1A), a medium that allows both growth and sporulation. The most striking feature of these sections was a sharp boundary between an upper layer containing many tetrad and dyad asci and a lower layer completely devoid of asci (Figure 1B). This boundary provides one of the clearest examples of cell patterning within the fungal kingdom.

FIGURE 2.—Sporulation initiates in central region of colonies. (A) A longitudinal section through the center of the colony, with the top (“T”) and bottom (“B”) of the colony indicated. To the left of the colony are 10 equally spaced marks that subdivide the colony into nine regions. Arrows indicate representative tetrad asci (four spores) and arrowheads indicate representative dyad asci (two spores). Bar, 50 μ m. (B) Sporulation distribution in 6-day colonies. Bars represent percentage of sporulation in each of the nine regions of each image, as shown in A, from the top of the colony (left) to the bottom (right) ($n = 4$). (C) Timing of growth and sporulation in colonies. Colony diameter (open triangles) and percentage of sporulation (solid circles) are shown in colonies (SH1020) grown for various times on YNA medium ($n = 5$).

Sporulation initiates in the interior of colonies and at the agar surface: The sharp boundary between sporulated and unsporulated regions led us to investigate the development of sporulation patterns after 4, 6, 8, and 10 days. Initially, four distinct layers of alternating sporulating and unsporulated regions were observed. For example, after 6 days, a thin layer of cells (3- to 5-cell widths) at the bottom of the colony contained a high frequency of tetrad and dyad asci (region 9 in Figure 2, A and B). Above this layer was a second layer (25- to 30-cell widths) that contained few if any asci (region 4–8 in Figure 2, A and B). Above this was a third layer, which was of variable size (5- to 10-cell widths) and contained many asci (region 2–3 in Figure 2, A and B). Finally, at the top of the colony was a layer of cells (5- to 10-cell widths) that contained relatively few spores (region 1–2 in Figure 2, A and B).

The interior region of sporulation expands as colonies mature while colony size remains constant: As colonies matured from 4 to 10 days, the interior layer

FIGURE 3.—Time course of spore distribution in wild-type colonies. Wild-type colonies were sectioned after 4, 6, 8, or 10 days on YNA medium, and the spore distribution was analyzed as in Figure 2. A single colony in the height range of 0.8–1.0 mm was analyzed at each of the indicated times.

of sporulated cells expanded upward to include the top of the colony (Figure 3). In contrast, the lower band of sporulated cells remained relatively narrow even after 10 days. Despite the expansion of the interior sporulation layer, the boundaries between the interior layer of unsporulated cells and the two surrounding layers of sporulated cells remained sharply defined, even after many days of incubation (Figure 3).

It is possible that the changes in sporulation patterns over time described in the previous paragraph result from ongoing cell growth in the colony. However, we found that the diameter of colonies remained constant after 4 days of incubation, and spores began to appear only after this time (Figure 2C). Similarly, the average number of cells per colony did not increase significantly from 4 to 6 days ($P = 0.82$, $n = 3$). Thus, it is unlikely that continued cell division plays an important role in the development of sporulation patterns in colonies.

Patterns of *IME1-LacZ* and *IME2-LacZ* expression in colonies: Sporulation in specific regions of colonies could reflect nonuniform expression of sporulation regulators through the colony. Two key activators of sporulation, the Ime1p transcription factor and the Ime2p protein kinase, are not always coregulated, because *IME2* expression requires Ime1p-independent signals as well as Ime1p (reviewed in HONIGBERG and PURNAPATRE 2003). To investigate the expression patterns of *IME1* and *IME2* within colonies, we examined colony cryosections expressing either *prIME1-LacZ* or *prIME2-LacZ* promoter fusions. For the initial experiments, we measured *prIME1-LacZ* in an *ime1Δ* background and *prIME2-LacZ* in an *ime2Δ* background to separate induction of these genes from autoregulation by their products (*i.e.*, feedback). Strikingly, the pattern

FIGURE 4.—Expression of *prIME1-LacZ* and *prIME2-LacZ* in colonies. Colonies were sectioned after 12 days incubation on YNA medium containing X-gal. (A) *prIME1-LacZ/ime1Δ* strain (SH3830); (B) *prIME2-LacZ/ime2Δ* strain (SH3825). Bar, 0.1 mm.

of expression for the two fusion genes was different (Figure 4). After 12 days incubation, both genes were expressed in a narrow band at the bottom of the colony, but *prIME1-LacZ* was also expressed through a broad region from the top to the center of the colony (Figure 4A), whereas *prIME2-LacZ* was mainly expressed in a narrow band in the center of the colony (Figure 4B). Because the overlap between *IME1* and *IME2* expression domains corresponds to the region of the colony in which asci were first observed, the interior layer of sporulation in colonies may reflect a region apical enough to express *IME1* and basal enough to express *IME2*.

To investigate the development of *prIME1-LacZ* expression patterns over time, colonies were sectioned at 4, 6, 8, and 10 days. At 4 days, the *prIME1-LacZ* expression zone was limited to the top approximately one-fourth of the colony, but by 6 days this zone had expanded to include approximately the top half of the colony (Figure 5A). The same pattern of expression was observed in a *prIME1-LacZ/IME1*⁺ strain as in the *prIME1-LacZ/ime1Δ* strain (Figure 5C); this result indicates that *Ime1p* is not required for the pattern of *IME1* transcription in colonies.

Expression of *prIME2-LacZ* peaked several days after *prIME1-LacZ* expression (compare Figure 5A and 5B). Interestingly, the LacZ expression pattern was different in *prIME2-LacZ/IME2* strains relative to *prIME2-LacZ/*

FIGURE 5.—Time course of *prIME1-LacZ* and *prIME2-LacZ* expression patterns and requirement of *Ime1p* or *Ime2p* for these patterns. Colonies were incubated for the indicated times on YNA medium containing X-gal and sectioned, and the distribution of LacZ expression was quantified as described in MATERIALS AND METHODS ($n = 3$). Each bar in the distribution from left to right represents the average LacZ expression level in one of 25 bins from the top of the colony ("T") to the bottom ("B"). (A) *prIME1-LacZ/ime1Δ* strain; (B) *prIME2-LacZ/ime2Δ* strain; (C) *prIME1-LacZ/IME1* strain; (D) *prIME2-LacZ/IME2* strain.

ime2Δ strains (compare Figure 5B and 5D). Both strains expressed LacZ at the bottom of the colony, but *prIME2-LacZ/IME2* colonies (unlike *prIME2-LacZ/ime2Δ* colonies) expressed LacZ only transiently if at all in the interior of the colony. A likely explanation for this difference is that *prIME2-LacZ/IME2* cells rapidly repress the fusion gene as they complete sporulation (negative feedback), whereas *prIME2-LacZ/ime2Δ* cells continue to express this gene. Thus, the *ime2Δ* background allowed visualization of the initial expression of *prIME2-LacZ* in a central region of the colony and also allowed accumulation of the fusion gene product to higher levels.

Signaling within colonies: Higher expression of *prIME2-LacZ* in *ime2Δ* strains relative to *IME2*⁺ strains was apparent not only in cryosections (Figure 5, B and D) but also in whole colonies viewed from the top, including colonies inoculated from a drop of culture, termed spot colonies (Figure 6, A and B, compare i and ii; quantification shown in Figure 6C). This observation is consistent with previous studies showing that *IME2* is regulated through negative feedback (MITCHELL *et al.* 1990). Negative feedback occurs in part through cell autonomous mechanisms; for example, Ime2p targets Ime1p for degradation, thus repressing transcription of its own gene (GUTTMANN-RAVIV *et al.* 2002). In addition, negative feedback in the context of colonies could proceed through cell nonautonomous mechanisms; for example, signals secreted by one cell could regulate sporulation in neighboring cells.

To distinguish between cell autonomous and non-autonomous mechanisms, we mixed equal numbers of cells from two different strains (a “reporter” and a “signal” strain) and then spotted the mixture on plates to generate “chimeric” colonies. The reporter strain contained the *prIME2-LacZ* gene, whereas the signal strain lacked the fusion gene; so, if the genotype of the latter strain affects LacZ expression in the chimeric colony, then there must be communication between cells. We verified that the two strains in each chimera were present in an approximately 1:1 ratio after colony growth was complete by resuspending cells, plating for isolated colonies, and determining the genotype of these isolated colonies (see MATERIALS AND METHODS). As a control, *prIME2-LacZ/ime2Δ* reporter cells were mixed with equal numbers of *ime2Δ/ime2Δ* signal cells (Figure 6, A–C, iii). In these control colonies, LacZ expression in this colony was approximately half that observed in colonies containing only *prIME2-LacZ/ime2Δ* cells, as would be expected from the 1:1 ratio of genotypes (Figure 6, A–C, compare ii and iii).

We next examined *prIME2-LacZ* expression in chimeric colonies containing equal mixtures of *prIME2-LacZ/ime2Δ* and *IME2/IME2* cells (Figure 6, A–C, iv). If induction of sporulation is not affected by neighboring cells, then chimeras containing *IME2/IME2* signal cells should be the same intensity blue as chimeras containing *ime2Δ/ime2Δ* signal cells. In contrast, we found that the *IME2* chimeras expressed *prIME2-LacZ* at much lower levels than *ime2Δ* chimeras (Figure 6, A–C, compare iii and iv). Thus, the genotype of cells within a colony affects the phenotype of neighboring cells in the same colony (*i.e.*, a cell nonautonomous mechanism). The same result was also obtained when an *IME2*⁺ reporter strain, *IME2*, was substituted for the *prIME2-LacZ/ime2Δ* strain (Figure S1 A). These results indicate that cell-to-cell signals regulate *IME2* expression in colonies.

As an independent test of signaling within colonies, we measured spore formation in *IME2* + *ime2Δ* chi-

FIGURE 6.—Cell-to-cell signaling regulates sporulation efficiency in colonies. (A) Diagram represents chimeric colony experiments, in which *prIME2-LacZ* expression was compared in four colonies containing the following genotypes: (i) *prIME2-LacZ/IME2* (SH3824) alone, (ii) *prIME2-LacZ/ime2Δ* (SH3825) alone, (iii) equal numbers of *prIME2-LacZ/ime2Δ* and *ime2Δ/ime2Δ* (SH3883), and (iv) equal numbers of *prIME2-LacZ/ime2Δ* and *IME2/IME2* (SH3881). Z, *prIME2-LacZ/ime2Δ*; i[−], *ime2Δ/ime2Δ*; I⁺, *IME2/IME2*. (B) Colonies diagrammed in A grown for 6 days in YNA-2 medium. As a control (c), a LacZ[−] colony (SH3881) was also spotted. (C) Quantification of *prIME2-LacZ* expression in colonies as described above (*n* = 5).

meras. Although *ime2Δ* mutants do not sporulate, increasing the fraction of *ime2Δ* cells in these chimeras increased the efficiency of spore formation in the *IME2* cells (Figure S1 B). Thus, intercellular signals regulate both *IME2* expression and spore formation within colonies.

Alkaline pH as both cause and effect of sporulation in colonies: As described in the Introduction, an alkaline pH environment has been implicated as both a by-product of sporulation and a signal that stimulates sporulation (OHKUNI *et al.* 1998). Thus, alkaline pH is a candidate for a cell-to-cell signal that regulates sporula-

FIGURE 7.—Alkaline pH, mediated through the Rim101p/PacC pathway, regulates colony sporulation efficiency. (A) Effect of pH on sporulation levels of wild type (SH3881), *rim101Δ* (SH4375), and *rim13Δ* (SH4415). Colonies were grown for 6 days on YNA-2 medium buffered to the indicated pH, and the percentage of cells in colonies that formed spores was assayed by microscopy after resuspending the complete colony ($n = 3$). (B) Production of alkali in wild type (WT) and *ime2Δ* mutant grown for 1, 2, or 6 days on YNA-2 media containing the pH indicator, phenol red. Red color in and around the patches of cells indicates an increased secretion of alkali from this strain. (C) *RIM101* and *RIM13* required to respond to neighboring cells. As indicated, chimeric colonies contained a *prIME2-LacZ/ime2Δ* reporter strain that is *RIM+* (SH3825), *rim101Δ* (SH4378), or *rim13Δ* (SH4417) and signal strains that are either *IME2+* (SH3881) or *ime2Δ* (SH3883). LacZ expression was quantified after 12 days of growth to detect low *rimΔ* expression. Black bars indicate chimeras containing

IME2+ signal strain, and gray bars indicate chimeras containing *ime2Δ* signal strain ($n = 3$). (D) *CIT1* is required for alkali production. *CIT1* (SH3881) and *cit1Δ* (SH4436) strains were patched to phenol red indicator medium containing both glucose and acetate (SPS2-0.3) to allow growth of the *cit1Δ* strain and photographed after 2 or 6 days of incubation. (E) *CIT1* is required to stimulate sporulation in neighboring cells. As indicated, chimeric colonies contained *prIME2-LacZ/IME2* reporter cells (SH3824) plus either the *CIT1* or the *cit1Δ* strain described in D as signal cells. Black bars indicate chimeras containing *CIT1+* signal strain, and gray bars indicate *cit1Δ* signal strain ($n = 3$). To ensure approximately equal numbers of *cit1Δ* and reporter cells in the mature chimeras, all chimeras were grown on medium containing both glucose and acetate (SPS2-0.3). Even in this medium, growth of the *cit1Δ* mutant is less than that of the wild type, so generation of mature chimeras containing approximately equal numbers of wild-type and *cit1Δ* strains required inoculating colonies with a 1:6 ratio of *IME2* reporter to *cit1Δ* strain and a 1:10 ratio of *ime2Δ* reporter to *cit1Δ* strain; genotyping of 6-day chimeric colonies (see MATERIALS AND METHODS) revealed that all contained from 40 to 50% reporter cells.

tion in colonies. To test this hypothesis, we first compared sporulation in wild-type spot colonies grown on medium buffered in the range pH 6.75–8.5. Sporulation in colonies showed a sharp dependence on pH in the range 7.5–8.5 (open triangles, Figure 7A). This result indicates that pH limits sporulation in colonies, consistent with the hypothesis that sporulating cells stimulate sporulation in neighboring cells by secreting alkali.

Given that the *ime2Δ* mutant stimulates sporulation in neighboring cells more than does the *IME2+* control, we next asked whether this mutant produces more alkali than the control. For this purpose, we compared extracellular pH surrounding *IME2* and *ime2Δ* strains using a pH indicator, phenol red. This indicator becomes increasingly red in the pH range 7.0–8.0. After a 1-day incubation, the mutant generated slightly less alkali than the wild type, consistent with higher induction of metabolic genes in sporulation-competent cells relative to sporulation-defective cells (PRIMIG *et al.* 2000). In contrast, after a 2-day incubation, the pink color surrounding *IME2* and *ime2Δ* patches was almost indistinguishable, and after a 6-day incubation, *ime2Δ* patches were noticeably redder than *IME2* patches. Thus, the wild type initially produced more alkali than the mutant; but over the longer term, the mutant eventually produced more alkali (Figure 7B).

The *ime2Δ* mutant may produce more alkali than the wild type because *IME2* specifically inhibits alkali production or because cells arrested in meiosis produce alkali for a longer period than the wild type. To address these possibilities, we measured alkali production in three other mutants arrested in meiosis: *ime1Δ* mutants, which like *ime2Δ* mutants arrest at the initiation of meiosis; *hop2Δ* mutants, which arrest early in pachytene (LEU *et al.* 1998); and *ndt80Δ* mutants, which arrest later in pachytene (XU *et al.* 1995). As was seen for the *ime2Δ* mutant, after 6 days, the meiotic-arrest mutants produced significantly more alkali than the wild type (Figure S2 A). This latter result is consistent with the wild type ceasing alkali production once sporulation is complete; whereas the mutants, which fail to complete sporulation, continue to produce alkali. Interestingly, the *hop2Δ* and *ndt80Δ* mutants are somewhat less red on pH indicator plates than the *ime1Δ* and *ime2Δ* mutants (Figure 7B and Figure S2 A), so on the basis of this qualitative assay, it may be that mutants arrested early in meiosis produce more alkali than mutants arrested later in the program.

We next tested the ability of the meiotic-arrest mutants to stimulate sporulation in chimeras. Chimeric colonies containing any of these mutants as the signal strain induced *prIME2-LacZ* in the reporter strain to significantly higher levels than did a wild-type signal

strain (Figure S2 B). Given the correlation between alkali production and the ability to stimulate sporulation, we propose that production of alkali during meiosis stimulates sporulation in neighboring cells.

Rim101p and Rim13p are required for response to alkali regulation of sporulation in colonies: Because the Rim101p pathway responds to alkali and is required for sporulation, we tested the ability of mutants in the Rim101p pathway (*rim101Δ* and *rim13Δ*) to respond to neighboring cells. Rim101 is a transcriptional repressor that activates sporulation genes probably by repressing other transcriptional repressors, such as Nrg1 (LAMB and MITCHELL 2003; ROTHFELS *et al.* 2005); Rim13p is a calpain-like protease required for processing and activation of Rim101p (LI and MITCHELL 1997).

We measured the ability of *rim101Δ prIME2-LacZ* or *rim13Δ prIME2-LacZ* reporter strains to respond to neighboring cells in chimeric colonies (Figure 7C). As expected given the role of the Rim101p pathway in *IME2* transcription, expression of the reporter gene in these *rimΔ* mutants (Figure 7C, bars 3–6) was considerably less than for *RIM*⁺ strains (Figure 7C, bars 1 and 2), although this expression was still easily detectable in the mutants after extended incubation. To measure the ability of the *rimΔ* reporter strains to respond to neighboring cells we compared expression of the fusion gene in chimeras containing an *ime2Δ* signal strain, which produces more alkali (Figure 7C, gray bars) to chimeras containing an *IME2* signal strain, which produces less alkali (Figure 7C, black bars). We found that *rimΔ* reporter strains expressed the fusion gene to the same level regardless of whether the signal strain was *IME2* or *ime2Δ* (Figure 7, compare bar 3 to bar 4 and bar 5 to bar 6). These results contrast with the control chimera containing a *RIM*⁺ signal strain, where even after extended incubation the signal strain is expressed to significantly higher levels when the signal strain is *ime2Δ* relative to when it is *IME2*⁺ (Figure 7C, compare bar 1 to bar 2, $P < 0.003$). Thus, *rim101Δ* and *rim13Δ* mutants are defective in responding to neighboring cells.

As a further test of the role of the Rim101p pathway in responding to alkaline pH, we measured the effect of pH on sporulation in *rimΔ* mutants. If the Rim101p pathway stimulates sporulation by responding to extracellular alkali, then *rimΔ* mutants should be defective in the stimulation of sporulation by alkaline pH. We found that *rimΔ* mutants sporulated to levels significantly less than the wild type, regardless of the pH (Figure 7A). More importantly, whereas sporulation efficiency increased dramatically for the *RIM*⁺ strain over the pH range tested, pH had little or no effect on sporulation efficiency for either *rimΔ* mutant. This result is consistent with the Rim101p pathway being required for cells to initiate sporulation in response to an alkaline pH environment.

Alkali signaling in colonies depends on respiration: Sporulating cells secrete alkali, specifically bicarbonate,

as a by-product of respiration. To examine the relationship between respiration and cell-to-cell signals, we examined a mutant, *cit1Δ*, that fails to respire on acetate medium (KIM *et al.* 1986). Thus, we grew *cit1Δ* and control strains on medium containing both glucose and acetate, which allows the mutant to grow but not to respire. Not surprisingly, we found that the wild type produced more alkali than did the *cit1Δ* mutant (Figure 7D). This result allowed an independent test of the role of alkali production in stimulating colony sporulation. To perform this test, chimeric colonies were prepared that contained a *prIME2-LacZ/IME2* reporter strain and a signal strain that was either *cit1Δ* or *CIT1*⁺. We found that expression of the reporter was significantly lower ($P < 0.02$) in the *cit1Δ* chimeric colony than in the *CIT1* chimera (Figure 7E, bars 1 and 2). Similar results ($P < 0.04$) were obtained when the reporter strain was *prIME2-LacZ/ime2Δ* rather than *prIME2-LacZ/IME2* (Figure 7E, bars 3 and 4). Thus, the *cit1Δ* mutant is defective both in producing alkali and in stimulating sporulation in neighboring cells, consistent with our model that alkali produced by respiration is a cell-to-cell signal that stimulates sporulation in colonies.

Effect of alkaline pH and *rim101Δ* mutations on colony patterns: To examine the effect of pH on colony sporulation patterns, colonies were grown on standard medium for 3 days, and then the pH of the medium was raised by addition of NaHPO₄ buffer to a well created in the agar (see MATERIALS AND METHODS). After an additional 3 days, the overall level of sporulation in these colonies was higher than in control colonies [$26 \pm 5\%$ sporulation for the pH-adjusted colonies ($n = 4$) *vs.* $16 \pm 1\%$ sporulation for unadjusted colonies ($n = 6$)]. Notably, the region of sporulation in pH-adjusted colonies was much broader than in control colonies (compare Figure 8A and 8B). This result suggests that the upward expansion of the central region of sporulation in colonies is normally limited by extracellular pH.

We next measured colony sporulation patterns in a *rim101Δ* mutant. The pattern of sporulation in *rim101Δ* colonies, even after 8 days of incubation, was much different from that in the wild type. In particular, the region of sporulation in the mutant colony was limited to a narrow central region of the colony rather than extending through the entire top half of the colony as in the wild type (compare Figure 8C and 8D). These results indicate that Rim101p, like alkaline pH, regulates the expansion of the interior region of sporulation within colonies.

DISCUSSION

The key results reported in this study are as follows: (1) Spore formation occurs in a sharply defined layered pattern in colonies, as does *IME1* and *IME2* expression, (2) *IME2* expression and spore formation within colonies are regulated by cell-to-cell alkali signals, and (3)

FIGURE 8.—Effect of pH and Rim101p on sporulation patterns. (A and B) Addition of alkaline buffer expands sporulation region in colonies. Wild-type colonies (SH3881) were grown on YNA medium for 3 days under standard conditions before 1.0 M sodium phosphate buffer (pH 8.0) was added (A) or not (B) to a well at the center of the plate. Incubation was continued for 3 additional days, and then colonies were sectioned and analyzed for the distribution of sporulated cells in the colony as in Figure 2B ($n = 3$). (C and D) Rim101p is required for expansion of sporulation region in colonies. Either *rim101Δ* colonies (C) or wild-type colonies (D) were incubated for 8 days before colonies were sectioned and analyzed for the distribution of sporulated cells in the colony as in Figure 2B ($n = 3$).

the Rim101/PacC pathway responds to alkaline pH to regulate both the efficiency and the pattern of sporulation.

Our results suggest a model in which sporulating cells in colonies secrete alkali and this alkali in turn stimulates sporulation in neighboring cells. This model is consistent with an earlier finding that cells in sporulation cultures secrete bicarbonate (an alkali), and this bicarbonate stimulates sporulation in these cultures (OHKUNI *et al.* 1998). Bicarbonate is produced during sporulation as a result of respiratory metabolism of nonfermentable carbon sources, and it is likely that secretion of alkali is just one of several ways that respiration and nonfermentable carbon sources stimulate sporulation (TREININ and SIMCHEN 1993; JAMBHEKAR and AMON 2008). Our model is strongly supported by the finding that mutants (*ime1Δ*, *ime2Δ*, *hop2Δ*, and *ndt80Δ*) producing more alkali than the wild type stimulated neighbor cell sporulation more than the wild type, whereas a mutant (*cit1Δ*) producing less alkali than the wild type stimulated neighbor cell sporulation less than the wild type.

Since *IME1* and/or *IME2* are required for induction of most or all sporulation genes, it is worth considering

why *ime1Δ* and *ime2Δ* mutants ultimately produce more alkali than the wild type. Many metabolic genes, including genes required for respiration, are induced under sporulation conditions, but most of these genes are induced under these conditions even in MAT homozygous strains, which cannot initiate sporulation (PRIMIG *et al.* 2000). Certain respiratory genes are induced to higher levels in sporulation-competent strains than in sporulation-defective strains (PRIMIG *et al.* 2000), and this higher induction likely explains why at early stages of growth on plates, the wild type produces more alkali than the mutant. In contrast, once cells complete sporulation, metabolism ceases until spores encounter an environment that promotes their germination (JOSEPH-STRAUSS *et al.* 2007); hence, alkali production also would be expected to cease once cells complete sporulation. In summary, the higher production of alkali in meiosis-arrest mutants is very likely because these mutants produce alkali for a longer period than the wild type.

Alkali signaling between cells likely accounts for the upward expansion of the region of sporulation in colonies from a narrow initial band. This conclusion is based on the observations that addition of alkali to the medium accelerates the expansion of this band, whereas the *rim101Δ* mutant prevents this expansion. The Rim101 pathway is required for yeast and other fungi to respond to moderately alkaline pH environments (7.0–8.0); for example, this pathway allows pathogenic fungi to survive alkaline host environments (DAVIS 2009). Prior to this study, the Rim101 pathway had not been implicated in cell-to-cell signaling. Our results raise the possibility that pH signals are a general form of communication between cells. For example, pH signals may regulate the organization of extended colony-like structures termed “mats,” since a gradient of acidic pH (4.7–5.8) contributes to variation in cell adhesion through different regions of the mat (REYNOLDS *et al.* 2008). Furthermore, alkalinization is also a by-product of the periodic production of ammonia in colonies that triggers repeated cycles of growth and stasis in haploid colonies (PALKOVA and VACHOVA 2003).

The bottom layer of cells in a colony inhabits a unique nutrient environment close to the agar surface, which may explain the cells’ high sporulation rate relative to more apical cells. In contrast, initiation of sporulation within a narrow interior band of cells cannot be explained by a unidirectional diffusion of nutrients through the colony (TURING 1952). Possibly, this region represents the intersection of two opposing gradients; for example, a gradient of acetate may diffuse upward from the bottom of the colony while a gradient of oxygen diffuses downward from the top. Consistent with this view, *IME1* and *IME2*, which are subject to different nutritional controls (see Introduction), are initially transcribed in different regions: *IME1* at the top of the colony and *IME2* in the central part of the colony.

Because both *IME1* and *IME2* are required for sporulation, the initial pattern of sporulation observed in the central region of the colony implies that this region may be apical enough to express *IME1* and basal enough to also express *IME2*.

Cell-to-cell signals may be necessary for efficient sporulation within the suboptimal nutrient environment present in developing colonies. Indeed, by limiting meiosis and sporulation to a subset of cells in the colony, colony patterning may maximize the overall survival rate of the colony's population. In the wild, yeast strains outcross with genetically diverse strains, and even though these outcrosses are relatively infrequent, they result in an increase in the genetic heterogeneity of the population (JOHNSON *et al.* 2004; RUDERFER *et al.* 2006; DIEZMANN and DIETRICH 2009). Since communities of yeast in the wild could often contain genetically diverse strains, we speculate that clustering asci of different strains together within these communities would promote outcrossing.

The foundation of pattern formation in metazoans is the combination of long-range and short-range cell signals (IRVINE and RAUSKOLB 2001; KERSZBERG and WOLPERT 2007); thus, it is interesting that colony sporulation patterns likely involve both long-range gradients of nutrients and short-range cell-to-cell signals. Differences in nutrient microenvironments across the colony could cause sporulation to initiate in specific regions, and pH signals between cells would reinforce these differences to sharpen the sporulation pattern. Nevertheless, pH signals are probably not sufficient to explain the remarkably sharp boundaries between sporulated and unsporulated regions in colonies. By analogy to pattern formation in metazoans, precise boundaries in developing colonies may require multiple signals, including both diffusible and cell-surface ligands. In this respect, yeast colonies may preserve some of the simplest, yet most ancient and most fundamental, mechanisms of cell patterning.

We thank Misa Gray [University of Missouri–Kansas City (UMKC)] for strain construction, Jessica Kueker (UMKC) and Jennifer Baumler (UMKC) for help in developing the sectioning protocols, Jeffrey Price (UMKC) for use of equipment, Gerald Wyckoff (UMKC) for suggestions related to image analysis, Justin Fay (Washington University) for helpful discussions, and Daniel Gottschling (Frederick Hutchinson Cancer Center) for advice on colony cryosections. This work was supported by grants from the National Institutes of Health (R15GM80710 and R15GM80710-S1) to S.M.H.

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Communicating editor: J. ENGBRECHT

GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.109.113480/DC1>

The Rim101p/PacC Pathway and Alkaline pH Regulate Pattern Formation in Yeast Colonies

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DOI: 10.1534/genetics.109.113480

FILE S1
SUPPORTING INFORMATION

Detailed protocol for colony embedding: The following is a modification of a method for embedding yeast colonies for light and electron microscopy (1). After incubation of approximately 300 colonies on agar medium for the indicated time, an isolated colony (1-2 mm in diameter) and a small amount of the underlying agar medium was removed and placed on a microscope slide. Several drops of 2% agar (42°C) were placed on a microscope slide, the colony was immediately placed on the agar and then several drops of agar were placed on top of the colony. The resulting agar block was trimmed with a razor blade, and placed in a 3.5 ml borosilicate screw-cap vial (Fisher 03-339-21B). All subsequent incubations and washes used 1.5 -2.0 ml and were performed in the same vial. First, colonies were fixed by incubating in 2% paraformaldehyde / 2% glutaraldehyde for 7 days at 4°C. Agar blocks were then washed on ice by incubating twice for 15 minutes with 0.5 ml of 0.15M sodium cacodylate (pH 7.2), and then twice more for 5 minutes with OS buffer (100 mM KH₂PO₄, 10 mM MgCl₂, pH 6.0). To allow electron microscopy of sections, 1% OsO₄ in OS was added to vials to cover the agar block and incubated on ice in a fume hood for 1 hr, then washed twice with OS buffer on ice, and incubated in OS overnight at 4°C. After washing two times with cold water, the blocks were washed sequentially with 25%, 50%, 75%, 95% and 100% (twice) ethanol for 10 minutes each, and the blocks left overnight at 4°C in 100% ethanol. Ethanol was removed, Spurr's reagent (Electron Microscopy Sciences) added, vials rotated for 15 minutes, then allowed to stand for 30 minutes, and the Spurr's treatment repeated four more times. After the final wash, Spurr's reagent was added to cover agar blocks (1.5-2.0 ml) and incubated for four hours, then the Spurr's was replaced, the vial rotated overnight, and this last step repeated again the next day. Finally, each agar block was placed in a mold with 0.2 ml of Spurr's and incubated at 60°C for four hours, topped off with Spurr's and incubated at 60°C overnight. Sections (0.5 µ) from the central region of the colony were collected in distilled H₂O, placed on a drop of water, and dried on a 52°C heat block. Sections were stained with 1.0 % toluidine blue, 1% Sodium Borate for 5-15 sec., washed under a stream of water, dried, covered in mounting media (KPL), and examined by light microscopy.

1. Scherz, R., Shinder, V. & Engelberg, D. (2001) *J Bacteriol* **183**, 5402-13.

Detailed Protocol for Cryosections: A square of agar surrounding a 2-3 mm colony was cut from an agar plate and submerged in OCT compound (VWR) in a 15 X 15 X 5 mm cryomold (VWR) and incubated at room temperature for 90 min. The cryomold was then submerged for 20 sec in an isopentane bath cooled by liquid nitrogen and sectioned at -20°C -- -25°C using a Microm HM 505E cryostat. 20 m sections through the central region of the colony were covered with mounting media (KPL) and examined using Nomarski optics.

FIGURE S1.—Effect of neighboring cells on *prIME2-LacZ* expression and spore formation in *IME2* cells. (A) LacZ expression in an *prIME2-LacZ/IME2* strain (SH3824) that it was present at the indicated concentrations (12.5% to 100%) in chimeric colonies that also contained either an *ime2Δ* strain (yellow) or an *IME2* strain (blue) or in a control colony containing only SH3824 (red). Particularly in the 25% chimera, there was significantly higher expression of LacZ in the chimeras containing the *ime2Δ* strain than the corresponding chimeras containing the *IME2* strain (for 25%, $P=0.02$; for 12/5%, $P=0.08$). (B) Spore formation in *IME2/IME2* + *ime2Δ/ime2Δ* chimeric colonies. Chimeric colonies inoculated at the indicated percentage of WT cells were examined for the percentage of cells that had formed spores after 5 days (circles) or 8 days (triangles) of incubation. The percentage of *IME2* cells that had sporulated (y-axis) was calculated by dividing the total percentage of sporulation in the colony by the percentage of *IME2* cells among total viable cells in the colony. This latter percentage was determined by suspending cells from each of three colonies, plating each suspension separately, patching approximately 60 of the resulting colonies from each plate to master plates, replica plating the master plates to sporulation medium, incubating for three days, and finally replica plating the sporulation plates to medium containing canavanine. This drug selects from cells that have completed sporulation, hence are *IME2*.

A. **B.**

FIGURE S2—Sporulation-arrest mutants produce more alkali and stimulate sporulation in surrounding cells more than the wild type. (A) WT (SH3881), *ime1*Δ (SH3972), *hop2*Δ (SH4302), or *ndt80*Δ (SH4300) were patched to YNA-2 containing phenol red and images captured after 6 days. (B) Chimeric colonies containing a prIME2-LacZ/*ime2*Δ reporter strain (SH3825) and a signal strain that was wild type, *ime1*Δ, *hop2*Δ, or *ndt80*Δ (same strains as in (A)) or *ime2*Δ (SH3883). Images were captured after 6 days of incubation, and LacZ expression quantified as described in Methods.

TABLE S1

***Saccharomyces cerevisiae* strains used in this study¹**

Strain	Genotype
SH1020	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 leu2-3,112/LEU2 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 trp1-1 / trp1-3'Δ ura3-1 / ura3-1</i>
SH3824	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 LEU2/leu2-3,112 trp1-1/trp1-3'Δ ura3-1/URA3 prIME2-LacZ-TRP1 / IME2</i>
SH3825	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 leu2-3,112/leu2-3,112 trp1-1/trp1-3'Δ ura3-1/URA3 prIME2-LacZ-TRP1 / ime2Δ::LEU2</i>
SH3826	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 leu2-3,112/leu2-3,112 TRP1/trp1-3'Δ ura3Δ::LEU2/ura3-1 IME1/ prIME1-LacZ-URA3</i>
SH3830	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 LEU2/leu2-3,112 TRP1/trp1-3'Δ ura3-1/ura3-1 ime1Δ / prIME1-LacZ-URA3</i>
SH3881	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 URA3/ura3-1</i>
SH3883	<i>MATa/MATα ade2/ade2 can1::ADE2::CAN1/can1::ADE2::CAN1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 lys2(3'Δ)::HIS3:lys2(5'Δ)/LYS2 URA3/ura3-1 ime2Δ::LEU2/ime2Δ::LEU2</i>
SH3972	Isogenic to SH3881 except <i>LEU2 / leu2-3,112 TRP1/trp1-3'Δ ime1Δ ime1Δ</i>
SH4300	Isogenic to SH3881 except <i>LEU2 / leu2-3,112 TRP1/trp1-3'Δ ura3-1/ura3-1 ndt80Δ::URA3 / ndt80Δ::URA3</i>
SH4302	Isogenic to SH3881 except <i>TRP1 / trp1-3'Δ ura3-1/ura3-1 hop2Δ::URA3Δ/ hop2Δ::URA3</i>
SH4375	Isogenic to SH3881 except <i>ura3-1/ ura3-1 rim101Δ::URA3/ rim101Δ::URA3</i>
SH4378	Isogenic to SH3825 except <i>ura3-1/ ura3-1 rim101Δ::URA3/ rim101Δ::URA3</i>
SH4415	Isogenic to SH3881 except <i>ura3-1/ ura3-1 rim13Δ::URA3/ rim13Δ::URA3</i>
SH4417	Isogenic to SH3825 except <i>ura3-1/ ura3-1 rim13Δ::URA3/ rim13Δ::URA3</i>
SH4436	Isogenic to SH3881 except <i>ura3-1/ ura3-1 cit1Δ::URA3/ cit1Δ::URA3</i>

¹ All strains constructed for the present study