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Yeast colonies synchronise their growth and development

Zdena Palková* and Jitka Forstová

Department of Genetics and Microbiology, Charles University, Viničná 5, 12844 Prague 2, Czech Republic
*Author for correspondence (e-mail: zdenap@prfdec.natur.cuni.cz)

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SUMMARY

The ability to emit and receive signals over long distances is one of the characteristic attributes of multicellular organisms. Such communication can be mediated in different manners (by chemical compounds, light waves, acoustic waves etc.) and usually is reflected in the behaviour of the communicating organisms. Recently, we reported that individual yeast colonies, organised multicellular structures, can also communicate at long distance by means of volatile ammonia, which is produced by colonies in pulses separated by acidification of the medium. Here, we demonstrate that the colony that first reached the stage of intense ammonia production induces ammonia production response in surrounding colonies regardless of their age, causing the synchronisation of their NH_3 pulses and, consequently, the mutual affection of their growth. Also an artificial source of ammonia (but neither NH_4^+ nor NaOH gradients) can immediately induce the ammonia production even in the colony starting its acidic stage of the development. The repeated transition of *Candida mogii* colonies from the acidic phase to the phase of intensive ammonia production is accompanied by dramatic changes in colony morphology and also in cell morphology and growth. Relatively smooth colonies in the acidic phase are formed by growing pseudohyphae. After ammonia induction, pseudohyphae decompose into non-dividing yeast-like cells, which rearrange themselves into ruffled spaghetti-like structures. The synchronisation of colony growth, that also exists between yeast colonies of different genera, could be important in establishing their optimal distribution in a natural habitat.

Key words: Yeast colony, Ammonia signalling, Synchronisation, Colony morphology

INTRODUCTION

It is usual to divide organisms into two basic categories: unicellular and multicellular. However, looking at unicellular organisms living in natural conditions, one can see their clear tendency to form multicellular structures, biofilms (Stickler, 1999), colonies (Shapiro, 1995, 1998; Palková et al., 1997; Ben-Jacob et al., 1998) and fruiting bodies (Kaiser, 1998, 1999), where individual cells exhibit an organised behaviour, co-operate in nutrient degradation and differentiate into functionally specialised cells. Classic, well-studied models of the multicellular behaviour are represented by *Myxobacteriae* (Kaiser, 1998, 1999) or *Myxomycetes*, the most well known representative of which is *Dictyostelium discoideum* (Firtel, 1996; Soderbom and Loomis, 1998). Structures formed by these organisms are composed of non-dividing motile cells. Such cells, when starved of nutrients, start to secrete into their surroundings signalling compounds, e.g. cAMP, amino acids etc., which are able to induce the production and secretion of the same signalling compound in the neighbouring cells. In this way, the signal is spread throughout the whole area, causing the synchronisation of the cells, which consequently change their behaviour. They start to move into the aggregation centre and create an aggregate, which differentiates into a fruiting body. This mechanism ensures that the first impulse emitted as a response by cells monitoring the level of nutrients is spread into other cells before the nutrients are completely depleted. Another signalling system, involved in the multicellular behaviour of bacteria, is termed ‘quorum sensing’ and depends on the production of one or more diffusible signal molecules (termed ‘auto-inducers’ or ‘pheromones’), which enable a bacterium to monitor its own cell population density. Quorum sensing regulates diverse physiological processes including bioluminiscence, swarming, synthesis of antibiotics and production of virulence determinants in pathogenic bacteria. In Gram-negative bacteria, the best understood signal molecules are the N-acylhomoserine lactones or post-translationally modified peptides (Hardman et al., 1998). The principle of auto-induction allows a more efficient increase in the signalling compound level than that which could be achieved by the increase in numbers of non-induced cells. Thus, the population can change its behaviour before reaching the critical density.

A yeast colony, a multicellular structure with a characteristic organised morphology, is formed during the divisions of non-motile cells. Organisation of yeast colonies should therefore be ensured by signals transmitted and received by dividing cells within the colony. Such short-range signals might also determine the polarity of cell division within a colony. In this regard, the yeast colony exhibits an analogy to embryogenesis of higher organisms, where the determination of the polarity of cell division plays a key role in the ontogenesis.

One of the characteristic attributes of multicellular organisms is their ability to emit and receive signals over long distances. Such communication can be mediated by chemical compounds, light or acoustic waves etc., and usually is reflected in the behaviour of the communicating organisms. We discovered recently that yeast colonies use for the long-range, inter-colony signalling a simple volatile compound, ammonia, produced by colonies in pulses (Palková, 1997). The first pulse of ammonia was found to be non-directed and followed by acidification of the medium. The second pulse seemed to be enhanced, oriented towards the neighbouring colonies and dependent on the presence of amino acids in the medium. NH_3 signalling resulted in asymmetric growth of the colonies, expanding into the free area (Palková et al., 1997). Ammonia was previously described as a signalling molecule involved in multicellular development of *D. discoideum*, where it plays a role in spore differentiation and pseudoplasmodium culmination, keeping *Dictyostelium* in the slug stage (Gross, 1994). In higher eukaryotes, ammonia or/and ammonium was shown to be the important signalling molecule of neurones, exhibiting there a large variety of biochemical and neurological effects and being also involved in many pathological situations. Ammonia has a role in normal metabolism, but when present in excess, it can disturb reversible reactions in which it participates, causing symptoms of 'hyperammonemias' connected with several serious diseases (e.g. Alzheimer type II astrocytosis in the adult, congenital urea cycle disorders or Reye syndrome of the infant, accompanied by cerebral atrophy and neuronal loss; Szerb and Butterworth, 1992; Butterworth, 1998). Examples mentioned above imply that ammonia could function as a universal signalling molecule in different organisms including mammals. The elucidation of the mechanism of its action on yeast colonies could help understand analogous processes in higher eukaryotes.

Here, we address how the ammonia signalling system operates when colonies of different age and in different phases of development are located near each other, the situation usual in natural conditions.

MATERIALS AND METHODS

Medium and yeast strains

GM-BKP agar (1% yeast extract, 3% glycerol, 2% agar, 30 mM $CaCl_2$, 0.01% bromocresol purple).

Strains of *Saccharomyces cerevisiae* GRF18 (α, his3, leu2), *Candida mogii*, *Cryptococcus albidus*, *Rhodotorula glutinis*, *Kluyveromyces marxianus* and *Schwanniomyces occidentalis* were from the Collection of Yeast Cultures of the Department of Genetics and Microbiology (DMUP), Charles University, Prague.

Induction of ammonia production by artificial ammonia source

Ammonia was generated in a small vessel containing NH_4Cl (70 mg) and 1 M NaOH (70 μl). The vessel was placed on the plate as shown in Fig. 2a, at different times of colony development, for approximately 15 minutes to enable the formation of an NH_3 gradient. During this period a gradient of pH was manifested by a violet colour which appeared on the surface of the agar medium precisely above the NH_4Cl/NaOH containing vessel. After quickly removing the ammonia source, the colony was exposed to the ammonia atmosphere maintained by continual evaporation of ammonia dissolved previously in the agar. This arrangement simulated a moderate ammonia release mediated by an inducing partner colony.

Gradient formation in the agar

Gradients of either NH_4Cl or NaOH were produced by diffusion from holes formed in the agar at different distances (1.0, 1.5 and 2.0 cm) from a growing *C. mogii* colony. 100 μl of 300 mM NH_4Cl and 100 μl of 300 mM NaOH were placed into the holes.

Diamino phenylindol (DAPI) staining of yeast cells

Cells were picked up from distinct parts of a colony, resuspended in DAPI solution in 10% ethanol (0.5 μg/ml) and examined under a fluorescence microscope in UV light (Olympus WIG filter).

Photography

Colonies were photographed either with illuminating light coming through a plate from the bottom (Figs 1B, 2b, 6) or they were illuminated from above (Figs 3, 4). A colour camera HITACHI HV-C20 with either Cosmicar or Navitar objectives, Fiber-Lite PL-800 and Kaiser Prolite illumination systems and Lucia G/F software (Laboratory Imaging s.r.o., Prague) were used.

RESULTS

Candida mogii giant colonies synchronise their 2nd pulse of ammonia production

We inoculated 7 GM-BKP agar plates (containing pH dye indicator) with 7 solitary giant colonies (Kocková-Kratochvílová, 1990; Palková et al., 1997) of *Candida mogii* and incubated them in parallel with 7 GM-BKP plates bearing no colonies ('empty' plates) at 28°C. At the time points indicated in Fig. 1, a new giant colony ('young' colony) was inoculated either near the 'old' one (a), or as a solitary colony on the empty plate (b). The young colonies exhibited the first alkali pulse shortly after inoculation, independent of the phase of development of the old colony. On the other hand, the second pulses of ammonia production of young colonies were influenced by the presence and stage of development of their old neighbours. When the young colonies were inoculated near the old ones with less than a 6-day delay, i.e. at the time when the old colonies were in the acidic phase (before the initiation of the second alkali pulse), the young colonies were still able to initiate their acidic phases (Fig. 1aI-VI). However, these phases were significantly shortened and colonies responded by ammonia production immediately after the old colonies had started their second alkali pulse (e.g. Fig. 1aVI). When the young colony was inoculated near the old colony which had already entered its second alkali phase, the acidic phase of the young colony was completely eliminated (Fig. 1aVII). The young colony responded with an intense and oriented alkali pulse. When the ammonia production of the young colonies was induced, the growth of their borders proximal to the old colony became inhibited. On control empty plates (bearing no old colonies), the young colonies exhibited the normal timing of pH pulses and symmetrical growth (Fig. 1b). These results suggest that colonies of *C. mogii* can change their metabolism from the 'acidic' to the 'alkali' phase as a response to the ammonia signal coming from the 'older' colony.

Ammonia is responsible for the synchronising effect

To confirm that ammonia is the agent responsible for synchronising the colony development and to exclude the

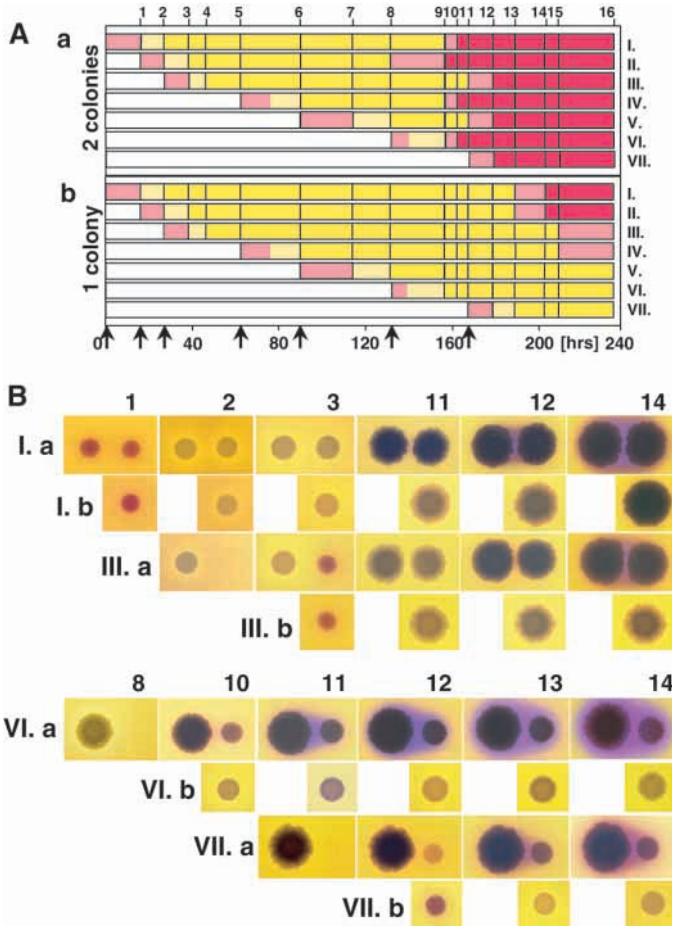

Fig. 1. Synchronisation of the development of giant colonies of *C. mogii*. (A) Diagram of pH changes around young colonies growing on GM-BKP agar either in the proximity of the old colony (a) or as solitary colonies (b). The time points of inoculation of young colonies are indicated by arrows at the bottom of the diagram. The old partner colonies were inoculated at the time zero. The numbers at the top of the diagram indicate the points on the time scale when photographs were taken. Bromocresol purple changes colour from yellow (below pH 5.2) to violet (above pH 6.8). (B) Examples of photographic documentation. Data are representative of three experiments.

possibility of the involvement of additional metabolites diffusing within the medium from the neighbouring colony, we induced the second alkali pulse, placing an artificial ammonia source near the colonies of *C. mogii* of different ages. The colonies responded by efficient ammonia production, regardless of the phase of their development. Fig. 2 shows an example of induction of a *C. mogii* colony which is in its acidic phase. In order to distinguish whether the active component is NH_3 or NH_4^+ or just the alkali gradient, we tried to induce the transition of colonies from the acidic phase of development to the phase of ammonia production by gradients of NH_4Cl and NaOH, respectively. In contrast to the ammonia gradient, neither ammonium chloride nor sodium hydroxide gradients expanding from the hole in the agar, situated in the proximity of the *C. mogii* colony, induced the acidic-phase colony to produce ammonia (data not shown).

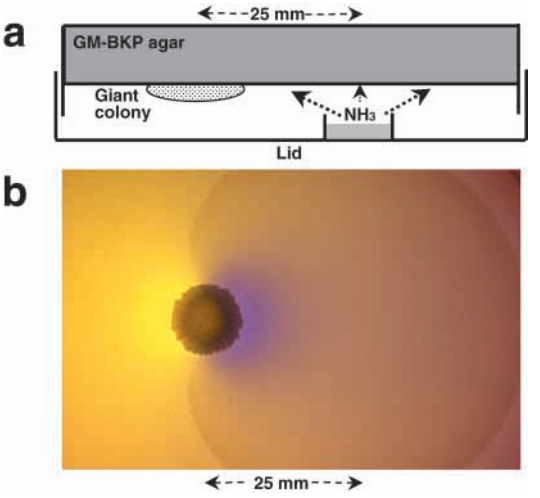

Fig. 2. Artificial ammonia source induces ammonia production in a *C. mogii* colony which is in the acidic stage of the development. (a) Diagram of the experimental arrangement. (b) The ammonia source was placed near a 3-day-old giant colony at the distance as indicated. The photograph was taken 18 hours later.

Induction of *C. mogii* giant colonies was accompanied by expressive changes in their morphology

When *C. mogii* giant colonies were induced to their own intensive ammonia production by the ammonia either produced by a partner colony (Fig. 3A), or released from the artificial source (data not shown), their morphology changed dramatically. A relatively 'smooth' colony in the acidic stage of development (Fig. 3Aa,Ba,b) started to change in area, responding with strong ammonia production, and by acquiring a ruffled 'spaghetti-like' structure (Fig. 3Ab,c,e). These changes gradually spread throughout the whole colony and were accompanied by transition of cells from pseudohyphal (Fig. 3Ad) to the yeast-like form (Fig. 3Ag). Because only one nucleus in each yeast-like cell was found by DNA staining (DAPI; Fig. 5Ba), we could conclude that these cells did not divide and the growth of the colony became arrested during its ammonia production. With the decline of the peak of ammonia production, the ruffled spaghetti-like structure gradually deflated (Fig. 3Bc,d), the colony started to grow again and entered the second acidification stage. It again became smooth, formed by pseudohyphae (Fig. 4a). The third ammonia pulse of *C. mogii* colonies started in the cells situated on top of the facing areas of the partner colonies (Fig. 4e) and was again accompanied by spaghetti-like rearrangement and pseudohyphae decomposition. In this stage of development, both partner colonies already exhibited substantial asymmetry of their shape.

To estimate the time of the cell proliferation arrest caused by ammonia induction, we followed the cells located in the border area of a *C. mogii* colony, which was proximal to the partner colony and which first became induced (Fig. 5). At the moment (time 0) when the border of the induced colony started to produce ammonia (became violet) and the first morphological changes appeared, the cells were found to be in the yeast-like form and no cells in the mitosis phase were visible (Fig. 5Ba). At the same time, the reverse side of the

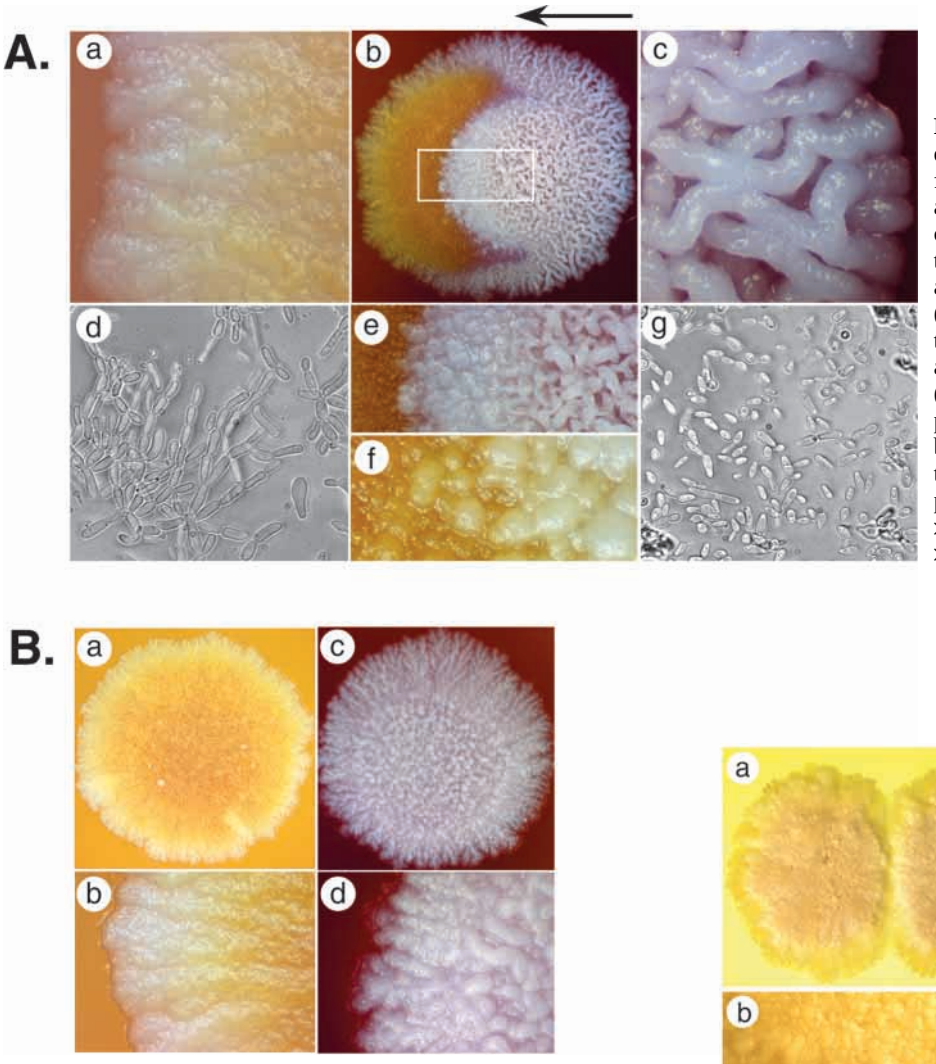

Fig. 3. Colony and cell morphology changes during the transition of a *C. mogii* colony from the acidic phase to the alkali stage of ammonia production response. (A) Giant colony induced by ammonia incoming from the right side (b); magnified details of the acidic left border (a) and alkali right border (c) and the region (in the white frame) where the first changes started (e,f). Cells from the acidic (d) and alkali (g) borders of the colony. (B) The morphology of a colony in the acidic phase of the development (a) and its enlarged border (b); a colony with deflated structure in the phase following the peak of ammonia production (c,d). Magnification: ×4 (B a,c), ×5 (A b), ×14 (A e), ×20 (B b,d), ×35 (A a,c), ×600 (A d,g).

colony (yet acidic) was formed by hyphae and pseudohyphae (Fig. 5A). Two days later, when alkali transition spread throughout the whole colony, the vast majority of the cells from the examined area (proximal to the partner colony) were still arrested in growth and only very sporadic mitoses were observed (Fig. 5Bb). One day later, when the ruffled structure of the colony deflated, many dividing cells, still retaining their 'yeast-like' shape, were observed (Fig. 5Bc). On day 6, prolonged cells, suggesting the formation of pseudohyphae, were visible (Fig. 5Bd). Three days later (day 9), pseudohyphae and hyphae were again re-established (Fig. 5Be). At that time the colony remained violet, not entering into an intensive acidification phase yet. The cell-growth arrest of the distal parts of the partner colonies was both substantially shorter and incomplete (not all pseudohyphae were found to be decomposed in the examined period; data not shown).

Synchronisation of colony development by ammonia signalling is a universal phenomenon

We were also interested in whether the synchronisation of colony development by ammonia signalling is a universal phenomenon between the colonies of different yeast genera exhibiting different growth properties. We inoculated two giant colonies of *C. mogii*, *R. glutinis*, *C. albidus*, *S. cerevisiae*, *K.*

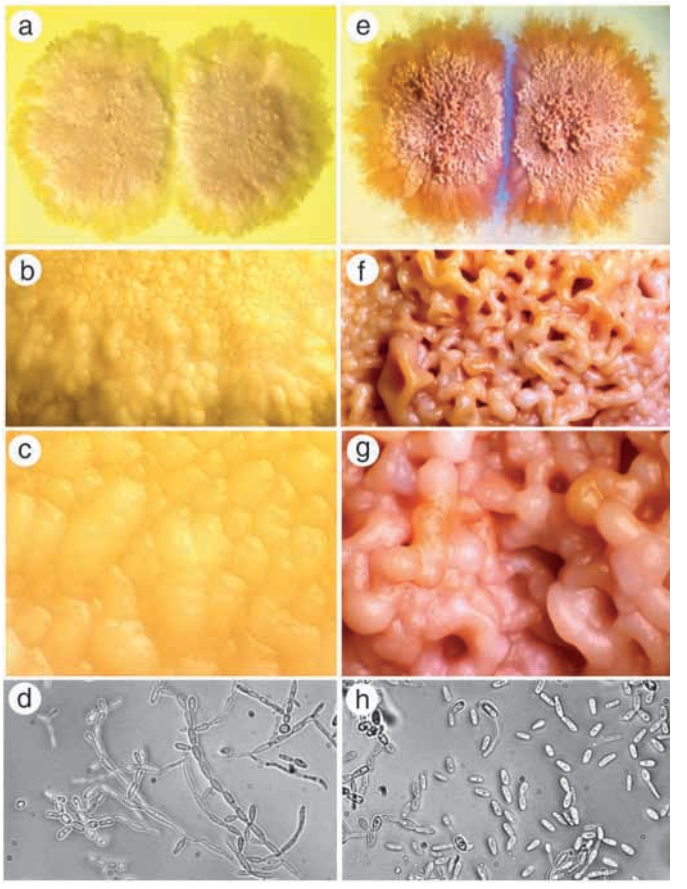

Fig. 4. *C. mogii* colony and cell morphologies in the 2nd acidic phase (a) and during the transition to the 3rd alkali phase (e) of the development. Enlarged details of the colony morphologies (b,c,f,g); the cells from the surface of the central part of the colonies (d,h). Magnification: ×2 (a,e), ×10 (b,f), ×30 (c,g), ×600 (d,h).

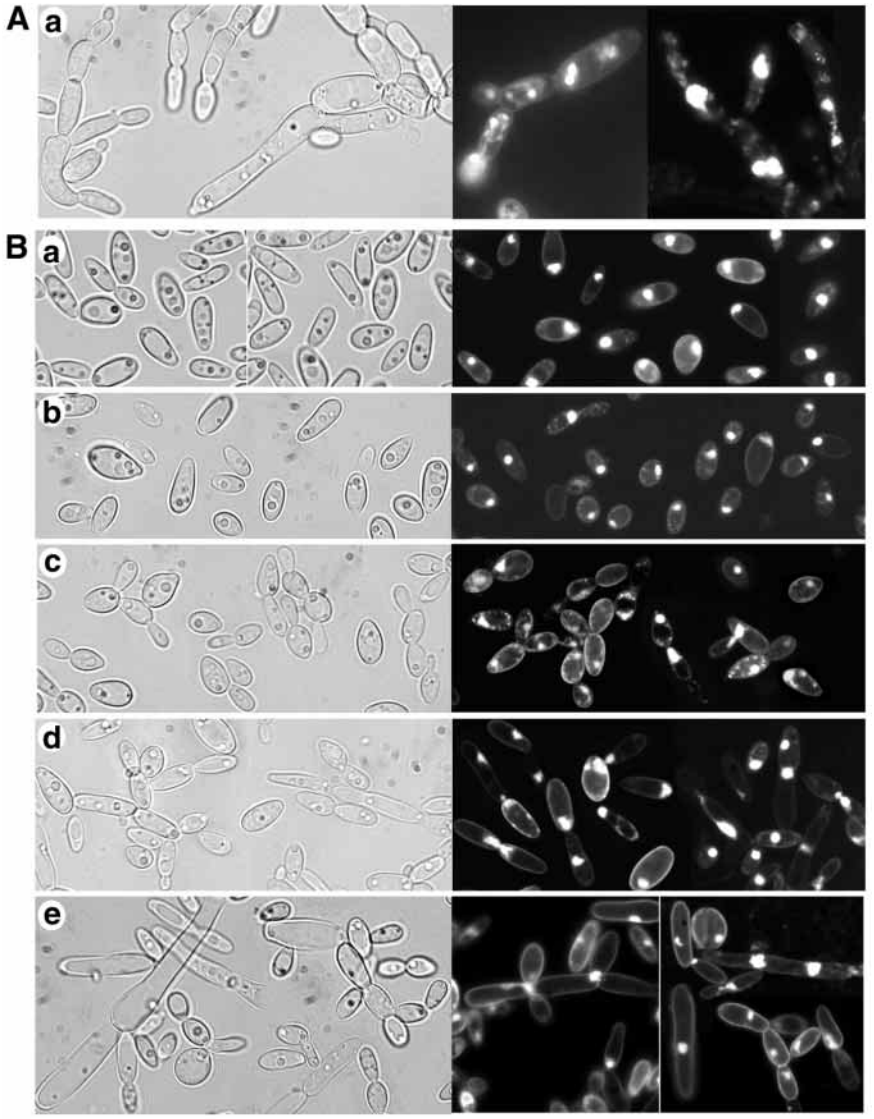

Fig. 5. Cells from non-induced (A) and NH_3-induced (B) areas of the *C. mogii* colony. Cells were analysed at the time when changes in the colony morphology appeared (time 0) (a) and 2 days (b), 3 days (c), 6 days (d) and 9 days (e) later. Cells under a light microscope (left panel); cells stained with DAPI (right panel). Magnification: ×1500.

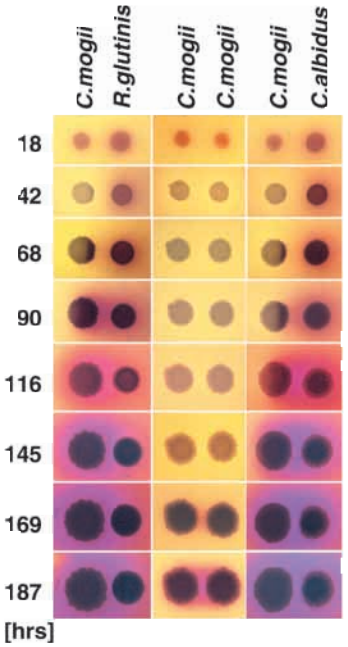

Fig. 6. Induction of ammonia production and synchronisation of giant colonies of different genera. Colonies of *C. mogii*, *C. albidus* and *R. glutinis* were inoculated at the time zero and photographed at the indicated time points.

marxianus and *S. occidentalis* in various combinations on GM-BKP medium. An efficient alkali switch of a *C. mogii* colony was induced by the presence of a colony of either *R. glutinis*, or *C. albidus* (Fig. 6). Colonies of both these species exhibited a more intensive first alkali pulse (in comparison with *C. mogii* colonies). This pulse was not followed by intensive acidification before the second, enhanced ammonia production. A *K. marxianus* colony, which exhibited the second pulse of ammonia production earlier than *C. mogii* colonies, evoked earlier and efficient induction of *C. mogii* colonies (data not shown). Also, a giant colony of *S. cerevisiae* was induced to produce ammonia several days earlier, when the partner colony was either a colony of *C. mogii* or *K. marxianus* (data not shown). These results imply that, irrespective of their taxonomic rank, yeast colonies co-ordinate their growth according to the colony which first entered the stage of strong ammonia production.

DISCUSSION

We have demonstrated that yeast colonies can change their metabolism from the phase of acidification of the surrounding medium to the alkali phase of intensive ammonia production, as a response to the ammonia signal incoming from an old colony which had already reached the stage of ammonia production. In this way, colonies in a particular area synchronise their ammonia pulses and their further development is co-ordinated. The induction of metabolic changes is a relatively fast process (a few hours), indicating a quick reaction of the colony to the presence of a competing colony in the neighbourhood. We have also noticed that the intensity of ammonia production (detected by pH indicator changes) by the larger old colony was enhanced and speeded by the presence of a young colony in its neighbourhood (Fig. 1). This supports our previous hypothesis of mutual enhancement of the second NH_3 pulses in neighbouring colonies (Palková, 1997) and implies a ‘ping-pong’ response pattern: volatile ammonia produced by a colony is immediately recognised by a neighbouring colony, which becomes induced to switch its metabolism to ammonia production and consequently enhances the ammonia production of the partner colony. This mechanism can explain the quick, enhanced and oriented start of alkali changes between two or several neighbouring giant colonies and relatively slow development

of alkali around a solitary colony. The observation that the time at which the first ammonia pulses of young colonies occur is not influenced by the presence of the old colonies agrees well with our previous observation that the first ammonia pulse is independent on the medium composition and on the presence of other colonies around (unpublished results). It could be therefore suggested that the first ammonia release, which starts immediately after the yeast cells are inoculated into new growth conditions, might originate from the protein turnover and/or from the internal amino acid pool.

When colonies of different genera, exhibiting different growth properties, were growing in the neighbourhood of each other, their development was also synchronised. In general, the colonies of species that earlier reached the phase of ammonia production induced the ammonia response in colonies of other species growing in their proximity. These results confirmed that, irrespective of their taxonomic rank, yeast colonies co-ordinate their growth according to the colony which first appeared in the stage of strong ammonia production.

The transition of *C. mogii* colonies to the phase of intense ammonia production could be induced, regardless of the age of the colonies, by an artificial ammonia source. Colonies in the phase of intensive acidification required a higher ammonia dosage for efficient induction than those in the phase of the first ammonia production or already reaching the beginning of the second alkali phase. The difference in sensitivity could be easily explained by the 'protective' effect of the acidic surrounding of the colony, leading to an immediate protonising of the incoming NH_3 to NH_4^+. Gradients of NH_4Cl or NaOH, expanding from holes in the agar situated in the proximity of a growing *C. mogii* colony did not switch its transition from the acidic to the alkali phase. These results are consistent with our hypothesis that the active molecule in the yeast colony communication is non-protonised ammonia. Previous observations (Bogonez et al., 1983) indicated that volatile NH_3, in contrast to NH_4^+, penetrates into yeast cells by simple diffusion through the cellular membranes. We propose that the mechanism of inter-colony NH_3 signalling might be similar to that suggested previously in *Dictyostelium discoideum* (Davies et al., 1993): NH_3 enters the acidic compartments (vacuoles) and becomes protonised. The resulting dissipation of the proton gradient between the vacuole and the cytoplasm then leads to the changes in cell behaviour.

Impressive changes observed during the induced transitions of *C. mogii* colonies from acidic to alkali phases (quick decomposition of pseudohyphae onto non-dividing yeast-like cells intensively producing ammonia and forming ruffled spaghetti-like structures) and the following reverse changes (when the colonies stop to produce ammonia, start to grow, form pseudohyphae and enter the next acidification phase) imply that colonies periodically switch from the phase of active growth (acidic phase) to the period of growth arrest. In arrested cells, intensive amino acid catabolism, accompanied by ammonia production, takes place, signalling to the neighbours the presence of other colonies. As the facing parts of the neighbouring colonies are induced first (and therefore inhibited longer than their opposite parts, situated in a free space), each ammonia pulse results in deeper asymmetry of the respective colonies forcing them to grow preferentially into the free space. The mechanism of switching on and off the ammonia production remains obscure and is being studied at present.

Our observations indicate that yeast colonies behave like real organised multicellular entities which are able, under suitable conditions, to communicate and co-ordinate their behaviour. The simple volatile compound, ammonia, as a signalling molecule, spreading efficiently through the air, represents a great advantage in natural conditions in which diffusion of the compounds forming gradients through a liquid can meet substantial restraints. Resembling the auto-induction mechanism leading to amplification of signalling molecules in bacterial populations (Kaiser, 1998, 1999), ammonia also induces its own production in the cells of exposed yeast colonies, causing quick changes and synchronisation of all colonies in the respective area. Moreover, such auto-induction is accompanied by inhibition of cell growth in the direction towards neighbouring colonies, which occurs before the nutrients are depleted. Therefore, this phenomenon, which is universal throughout the yeast genera, might have important consequences for establishing an optimal distribution of colonies of different genera in a natural habitat.

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