Variabilní genová exprese v buňkách kvasinkových kolonií

Differentiated Gene Expression in Cells within Yeast Colonies (MINARIKOVA *et al.* 2001).

Z dřívějších výsledků získaných v naší laboratoři vyplývá, že mnohobuněčné kvasinkové kolonie využívají signalizaci na dlouhou vzdálenost a jsou schopny kooperativního chování (PALKOVA and FORSTOVA 2000; PALKOVA *et al.* 1997). Kvasinková kolonie není pouhou neuspořádanou hromádkou buněk a její struktura vzniká analogicky jako tkáně vyšších organizmů dělením buněk. Musí proto existovat signály a geny které jsou důležité pro vývoj a morfologii kolonií.

Cílem naší práce bylo nalezení genů regulovaných či regulujících vývoj kvasinkových kolonií. Pro tento účel jsme vyvinuli metodu umožňující hledání genů variabilně exprimovaných v průběhu vývoje kvasinkové kolonie. Fragmenty genomové DNA *S. cerevisiae* jsme vložili před gen *lacZ* postrádající vlastní promotor. Část kolonií transformantů vykazovala modrobílý fenotyp, což nasvědčuje tomu, že před gen *lacZ* byl zařazen promotor střídavě zapínaný a vypínaný v průběhu vývoje kolonie. Následně jsme izolovali a identifikovali 5 genů s měnící se expresí během růstu kolonie: *CCR4, PAM1, MEP3, ADE 5,7* a *CAT2*. Pokud by některý z těchto genů byl důležitý pro regulaci vývoje kolonie, tak by jeho delece měla vést k poruchám vývoje. Ukázalo se, že deleční mutant *ccr4Δ* má oproti rodičovskému kmeni méně organizovanou morfologii kolonií, což nasvědčuje tomu, že by mohlo jít o gen důležitý pro vývoj kolonií. Složité vzorce exprese některých genů (např. *MEP3*) ukazují na to, že uvnitř kolonie existují subpopulace buněk s různou genovou expresí. Takže i u morfologicky zdánlivě jednoduchých kolonií laboratorního kmene *S. cerevisiae* dochází k diferenciaci buněk v průběhu jejich vývoje. Tomu nasvědčuje i naše pozorování synchronizované exprese některých genů v populaci kvasinkových kolonií.

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Differentiated Gene Expression in Cells within Yeast Colonies

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Yeast cells growing on solid media organize themselves into multicellular structures, colonies, exhibiting patterns specific for particular yeast strains. With the aim of identifying genes involved in regulations of the colony formation, we applied a new approach enabling the extensive screening of *Saccharomyces cerevisiae* genes, the expression of which is changed during colony development. We used the library of *S. cerevisiae* DNA fragments inserted in front of the *lacZ* gene lacking its own promoter. Colonies of transformants with a blue/white patterned morphotype, implying that the expression of the *lacZ* gene from the inserted yeast promoter is switched on and off during the colony formation, were isolated. We identified several genes with variable expression during colony morphogenesis, including *CCR4, PAM1, MEP3, ADE5,7* and *CAT2. S. cerevisiae* strain deleted in the *CCR4* gene forms colonies with less organized morphology when compared with the isogenic parental strain. The synchronization of the expression patterns of some of the isolated genes in neighboring colonies was observed. © 2001 Elsevier Science

Key Words: **yeast colony morphology; yeast cell differentiation; gene regulation; promoter library.**

INTRODUCTION

Development and differentiation of cells forming tissues of higher eukaryotic organisms are accompanied by the transmission of signals between individual cells and also between groups of specialized cells. Cell dedifferentiation leads very often to proliferation, tumor growth, and other pathological changes. Some unicellular organisms also behave, under appropriate growth conditions, as organized multicellular structures. *Myxobacteriae* and amebas of the slime mold *Dictyostelium discoideum* form organized fruiting bodies composed of nondividing motile cells [1, 2].

Yeast cells growing on solid agar medium are also able to create organized formations—colonies, morphologies of which are characteristic for individual species and strains. As the yeast cells are nonmotile, the colony pattern formation can be achieved only through the yeast cell division. Therefore, position information and intercellular signals determining initiation of the next cell division, the polarity of the growth, and the distribution of dividing cells within the colony, should exist. In this respect, the formation of a yeast colony exhibits similarities with the formation of specialized tissues of higher eukaryots.

At present, only limited data about the transfer of information and behavior of yeast cells within a colony are available. It was found that several strains of *Candida* sp. can "switch" between various types of colony morphology under different growing conditions. Cells from individual colonies with distinct types of morphology differ in shape, budding pattern, and adhesivity, and they exhibit changes in mRNA pattern [3]. The differences in adhesivity and invasive growth influence the virulence of clinical pathogen *Candida albicans,* budding yeast capable of forming a range of polarized and expanded cell shapes, from pseudohyphae to true nonconstricted hyphae. The process of so-called dimorphic transition from a budding to a filamentous form may allow the yeast to penetrate host epithelia and may therefore be important for the infection [4]. Meunier and Choder [5] showed that the early growth of *S. cerevisiae* colonies (followed for approximately 200 h) is biphasic, starting with exponential-like phase (approximately 24 cell divisions) and followed by a slow-growth phase occurring predominantly in a colony periphery. Scanning electron microscopy revealed that the cells in starved *S. cerevisiae* colonies are connected by fibrils forming a global network, which might mediate cell–cell communication [6].

We have shown that yeast colonies possess the ability to emit and receive signals at long distances, and we identified ammonia (produced in pulses) as the substance mediating the intercolony signal [7]. We also demonstrated that the colony which first reached the stage of intense ammonia production induces ammonia production response in the surrounding colonies, regardless of their age, causing the synchronization of their NH_3 pulses and consequently, their growth is mutually affected. The ammonia induction of *Candida mogii* colonies was accompanied by expressive changes

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in colony morphology and also in cell morphology and growth [8].

Identification of genes involved in the regulation of a colony development or genes regulated during colony morphogenesis might be a useful introduction to and a guide in the effort to understand developmental processes in the colony. Here, we describe a new approach for broad screening and identification of *Saccharomyces cerevisiae* genes which are switched on and off during the colony morphogenesis.

MATERIALS AND METHODS

Plasmid and strains. *S. cerevisiae* promoter library in the plasmid Yep366 [9] was kindly provided by M. Bolotin-Fukuhara (Universite de Paris-Sud, Orsay, France). *Saccharomyces cerevisiae* GRF18 (MATα, *his3, leu2*) was from the Collection of Yeast Cultures of the Department of Genetics and Microbiology (DMUP), Charles University, Prague. *S. cerevisiae* BY4742 (MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) and all isogenic mutants used in this work were from the EUROSCARF collection (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html).

Media. MMX (2% glucose, 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 54 mM KOH, 1.7 mM NaCl, 0.1% Wickerham vitamin solution [10], 0.16 mM histidine, 20 μg/ml X-gal), MMX-gln (MMX, 10 mM glutamine instead of 15 mM $(NH_4)_2SO_4$), MMX-glu (MMX, 10 mM glutamic acid instead of 15 mM $(NH_4)_2SO_4$), MMX-ala (MMX, 10 mM alanine instead of 15 mM $(NH_4)_2SO_4$), MMX-pro (MMX, 10 mM proline instead of 0.2% 15 mM) and YEPG (1% yeast extract, 2% peptone, 2% glucose, 2% agar) were used as media.

Transformation of S. cerevisiae with library and isolation of blue/white patterned colonies. Transformation of *S. cerevisiae* GRF18 by using the promoter library was performed as described [11]. Transformants were plated on MMX medium at a concentration of 20 to 100 transformants per plate and incubated at 28°C. The total number of transformants was 10,700. Appearance of colonies with a blue/white pattern was followed in the course of 3 to 4 weeks. The colonies were picked up and the cells replated on MMX agar, where they again formed 20 to 100 colonies per plate. The homogeneity of the morphotype of individual colonies was followed. If necessary, the replating procedure was repeated several times in order to obtain the colonies with uniform morphotype.

Isolation of plasmids from yeast transformants and their "purification" using E. coli. Plasmids from individual yeast transformants were isolated as follows. Briefly, yeast cells were disrupted with glass balls in LTEX buffer (2.5 M LiCl, 40% Triton X-100, 5 mM Tris–HCl, pH 7.5, 60 mM EDTA–NaOH, pH 8.0) and DNA extracted with phenol/chloroform treatment. Plasmids were precipitated with 98% ethanol, dissolved in TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA–NaOH, pH 8.0), and electroporated into *Escherichia coli* DH5α [12]. Plasmids from individual *E. coli* transformants were extracted and analyzed by using restriction enzymes [12]. Individual plasmids were retransformed into fresh *S. cerevisiae* GRF18 cells and plated on MMX medium. The morphotypes of original transformants and individual retransformants were compared in order to choose the plasmids responsible for particular morphotypes. If necessary, the procedure of plasmid isolation and retransformation was repeated several times.

Plasmid sequencing and database identification. Inserts of isolated plasmids were partially sequenced on the automatic sequencer VISTRA 725 (IM AVČR, Prague). The sequences of inserts were compared with sequences listed in the *Saccharomyces* Genomic Database (http://genome-www.stanford.edu/Saccharomyces/).

Chloroform treatment. The colonies (monocolonies or giant colonies) of transformants growing on MMX plates were exposed to chloroform vapor for 15 to 60 min. The colonies were further incubated at 30°C for another 24 to 48 h to develop additional coloring. The cell permeabilization by chloroform vapor was followed by using the methylene blue staining of cells picked up from exposed colonies.

Photography. Colonies were photographed with left and right illumination using two warm-light sources (60 W bulbs; Fig. 1A) or two cold-light sources (24 W fluorescent lamps; Figs. 1B, 1C, 3, 4, 5).

RESULTS

Identification of *S. cerevisiae* Transformants Creating Colonies of Two Colors, Blue and White

Promoter library of *S. cerevisiae* containing yeast genomic DNA fragments fused in two different reading frames with the coding sequence of the bacterial *lacZ* gene lacking its own promoter [9] was used for the transformation of *S. cerevisiae* GRF18. Transformants were grown on MMX selection medium containing X-gal chromogenic substrate, enabling the detection of colonies formed by transformants producing β-galactosidase, and therefore containing a plasmid with yeast regulatory sequences and a part of the original gene fused in proper reading frame with *lacZ* ("*lacZ* fusions"). As expected, after transformation with promoter library, we observed three basic types of colonies: (1) white colonies (w) that are formed by cells not producing β-galactosidase. These cells either do not contain plasmid with *lacZ* fusions, or the regulatory sequences on the plasmid do not permit the *lacZ* fusion expression during the colony development; (2) blue colonies (b) of transformants with a plasmid containing the promoter, which is active in all visible cells of a colony; and (3) colonies with a two-color pattern, white and blue, formed by transformants which might contain the *lacZ* fusion plasmid with a promoter which is switched on and off during colony morphogenesis (Fig. 1A). Blue/white colonies represented approximately 3% of all transformant colonies. In this way, several transformants were isolated and plasmids with a spectrum of promoters, the activities of which depend on the position of the cells within a colony at particular times of its development, were obtained.

Purification of *S. cerevisiae* Blue/White Patterned Colonies and Identification of Respective Genes

The blue/white colonies of transformants were picked up, and their blue/white morphotypes were verified by several replating of their monocolonies on MMX medium in order to eliminate those transformants which after replating segregate into colonies of different color morphotypes. Such originally blue/white colonies might arise either from more than one cell or from a transformed cell containing different plasmids irregularly segregating during cell divisions. The plas-

mids from transformants that formed colonies of homogeneous morphotype were next purified and retransformed onto fresh GRF18 yeast cells to prove the dependency of the colony pattern on the respective plasmid (Fig. 1B). This purification procedure was important because in some cases, several *lacZ* fusion plasmids were introduced into a single yeast cell during the process of primary transformation.

lacZ fusion inserts of five plasmids responsible for five particular blue/white morphotypes (Fig. 1B) were partially sequenced, and carried genes (*CAT2, PAM1, MEP3, CCR4,* and *ADE5,7*) were identified in the yeast genome database. Sequencing confirmed that all genes were fused with the *lacZ* sequences in the proper phase of translation. Mapping with various restriction enzymes proved the length and identity of a whole inserted fragment. Figure 2 shows the parts of the respective genes fused with the *lacZ,* the lengths of the upstream noncoding regions (thought to contain regulatory sequences), and an overlap of the fragments with other open reading frames (ORFs).

The expression of *lacZ* from the particular promoters was followed also in the giant colonies (colonies rising from more than one cell [7, 13]) of the respective transformants (Fig. 1C). Again, the colonies exhibited blue/white regular patterning, thus confirming our previous observation that the morphology of giant colonies is also specifically organized. In contrast to monocolonies, the blue/white morphotype of different giant colonies, growing on the same medium and formed by cells expressing β-galactosidase from one particular promoter of the library, exhibited higher variability (data not shown), although the basic characteristic were usually preserved. Also, the appearance of color sectors was more frequent in giant colonies when compared with monocolonies.

Sensitivity of the Detection System

The experience with *lacZ* system revealed that the sensitivity of the detection increases when the yeast cells producing β-galactosidase are permeabilized and the X-gal can enter the cells more efficiently [14]. In order to be able to follow weaker β-galactosidase expression, we tested various approaches of permeabilization, which would leave the structure of the colony undisturbed (e.g., freezing in liquid nitrogen, −70°C, application of chloroform, incubation at higher temperature). Several methods appeared to be useless (although the cell permeabilisation and X-gal staining were quite effective) because they led to a partial damage of the colony structure (e.g., freezing in liquid nitrogen; data not shown). The best results were obtained when colonies were exposed to vapors of chloroform for approximately 60 min and subsequently incubated at 30°C for 24 or 48 h. The intensity of the blue pattern of both monocolonies and giant colonies was significantly strengthened (Fig. 3A). The staining after chloroform treatment was intensified, particularly in those parts of colonies where original staining started to appear just before the chloroform application (usually the margin ring) (Fig. 3A). The "surface" cells of colonies were proved to be killed by chloroform vapors within 20 min (data not shown), thus avoiding possible artificial staining due to, for example, cell stress response.

Exploitation of lacZ Fusion Plasmids for Analyses of the Expression under Various Growing Conditions

Isolated plasmids containing *lacZ* fusions can be directly used as a tool for studies of the activity of carried promoters in colonies growing under various external conditions. Figure 3B shows the morphotypes of giant colonies formed by cells containing *MEP3-lacZ* fusion on media with four different amino acids (alanine, glutamic acid, glutamine, and proline) as the sole nitrogen source. The expression from the promoter of *MEP3* gene (encoding ammonia permease Mep3) in colonies growing on MMX-ala medium (Fig. 3B) differed from those growing on the MMX medium (containing NH_4^+, as a sole nitrogen source) (Fig. 1C). The promoter was active in cells of substantially larger area of the colony growing in the absence of ammonium. The expressions of *MEP3-lacZ* on MMX-glu and MMX-gln media were comparable and were induced mostly in the central areas of the colonies (Fig. 3B).

When *S. cerevisiae* giant colonies grew on medium with proline (poor nitrogen source) as a sole nitrogen source, their morphology was changed, and they formed many sectors (Fig. 3B). In contrast to expression of, for example, *PAM1,* which was not switched on during such sector formation (data not shown), *MEP3* exhibited different expression in different sectors (Fig. 3B). This indicates that the cells within a giant colony might differentiate under the stress conditions of a pure nitrogen source.

Blue/White Pattern Formation in the Neighboring Colonies of Different Size

In order to follow the activity of particular promoters in a population of colonies, the transformants were plated on MMX medium in different densities to form 10, 50, and 100 colonies per plate, respectively. The results suggested that the development of the expression pattern in the population of colonies was different for each of individual promoters (Fig. 4). With *ADE5,7,* the "central ring" expression pattern at the 20th day of a colony growth was identical in all colonies, regardless of their size; smaller (more crowded) colonies had the same numbers of blue and white rings (although they were narrower) as larger colonies. The patterns of col-

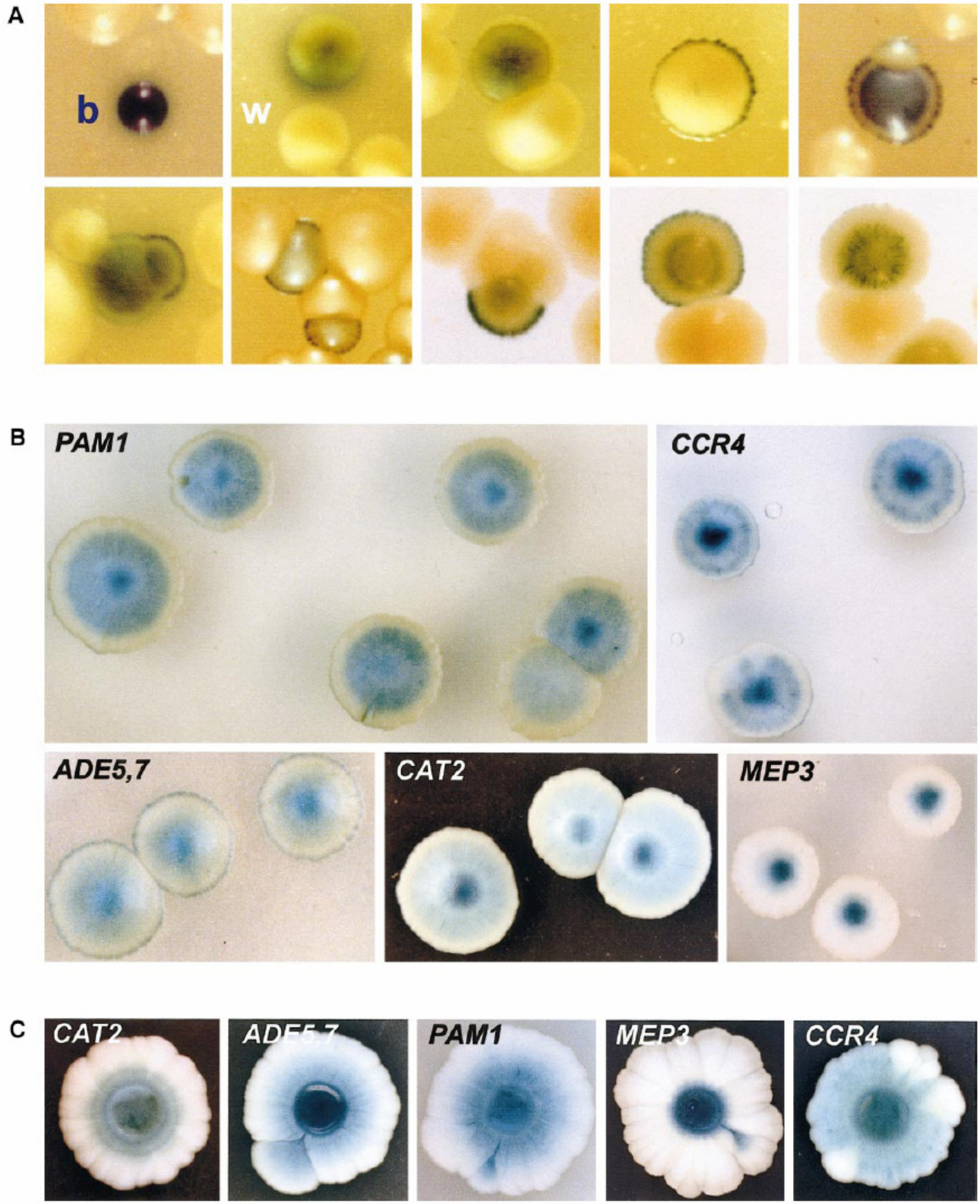


FIG. 1. Colonies formed by *S. cerevisiae* GRF18 transformed with *lacZ* promoter library. (A) Primary transformants grown on selection MMX medium for 15 to 30 days. b, blue colony; w, white colony. Magnification 3×. (B) Morphotypes of monocolonies of purified transformants. Monocolonies of transformants were grown on selection MMX medium for 22 days (*ADE5,7, CAT2, MEP3*) and 35 days (*PAM1, CCR4*) Magnification 3.5× (*ADE5,7*), 2.5× (*PAM1, CCR4, MEP3*) and 1.5× (*CAT2*). (C) Morphotypes of giant colonies of purified transformants. Giant colonies of transformants were grown on selection MMX medium for 20 days (*CAT2*) or 30 days (*ADE5,7, PAM1, MEP3, CCR4*). Magnification 1.5×.

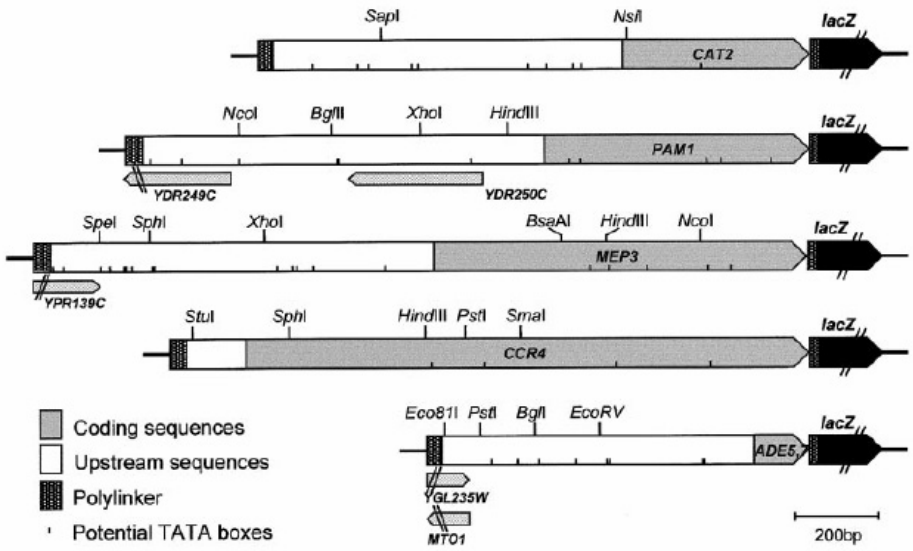


FIG. 2. The mapping of inserts of plasmids of the respective transformants. Inserts of *S. cerevisiae* genomic DNA in front of *lacZ* gene (black box) containing parts of *CAT2* (carnitine *O*-acetyltransferase), *PAM1* (coiled-coil protein and multicopy suppressor of loss of *Pp2A* phosphatase), *MEP3* (ammonium permease of high capacity and low affinity), *CCR4* (the component of the *Ccr4* transcriptional complex) and *ADE5,7* (glycinamide ribotide synthetase and aminoimidazole ribotide synthetase) coding and upstream sequences. Gray arrows indicate the ORFs overlapping with the respective fragments. Positions of restriction sites inside the fragments are as indicated. Small commas define the positions of potential TATA boxes.

onies of *CAT2* and *MEP3* transformants were also similar in small and large colonies, but in contrast to the *ADE5,7,* the central parts of all colonies exhibited the stained area of the comparable diameters, independently of the sizes of the whole colonies, while additional rings were wide in larger colonies and narrower in smaller ones. The expression patterns of *PAM1* colonies of different sizes were slightly different at later times of their development. In contrast to small colonies, central areas of larger colonies became blue at the 20th day. The expression pattern of *CCR4* was not properly manifested when the colonies were plated in higher densities. At the 20th day, the population of small colonies exhibited nonuniform staining (Fig. 4). The organized expression pattern of *CCR4* appeared on colonies that had already reached the larger size (Fig. 1B).

In contrast to the ring pattern appearance, which was quite uniform in most of the examples, the appearance of sectors was usually random and independent of the colony size and age (data not shown).

Colonies of *S. cerevisiae* Deleted in *CCR4* Gene Exhibit Changes in their Morphology

Colonies of strains deleted individually in identified genes were plated on various agar media in parallel with the isogenic parental strain. The morphologies of colonies formed by the strain deleted in *CCR4* gene differed from the morphologies of colonies of isogenic parental strain or other mutants (Fig. 5). The *CCR4* gene encodes a transcription factor, which is the component of the Ccr4 transcription complex and the component of the major cytoplasmic mRNA deadenylase. It has been shown to have both positive and negative effects on gene transcription [15].

DISCUSSION

We established the technique enabling the wide screening of genes, the expression of which varied during the development of colonies of *S. cerevisiae*. As most of the yeast DNA fragments carried on the *lacZ* fusion plasmids were large enough to contain all necessary regulatory sequences, and as their ability to regulate gene expression was analyzed in *S. cerevisiae* (the organism from which they originated), we can suppose that the regulation of *lacZ* gene expression might simulate that of the original gene on the yeast chromosome. Until now, we had identified five genes manifesting differentiated expression patterns in yeast colonies. *MEP3* encodes ammonium permease, transporting ammonium into the cells with high capacity and low affinity [16]. *ADE5,7,* the expression of which is induced on glucose [17], encodes two enzymes of purine metabolism [18]. *CAT2,* which was shown to be induced in the stationary phase and during nitrogen starvation [19], encodes carnitine acetyltransferase involved in the acetyl-CoA transport from peroxisomes to mitochondria [20]. Changes in expression of these

genes might reflect alterations of the cell metabolism in particular areas of a colony (e.g., due to nutrient concentration gradients). Different metabolic changes can consequently drive the further development of the colony. *PAM1* encodes a multicopy suppressor of the loss of Pp2A phosphatase, which, when overproduced, inhibits growth and induces the pseudohyphal phenotype [21]. *CCR4* encodes a transcription factor exhibiting pleiotropic functions (see above). Both Pam1 and Ccr4 might be involved in regulations of cell growth during colony development. Finding of morphology changes of colonies formed by a *ccr4* mutant supports this prediction.

Meunier and Choder [5] used an unstable version of *lacZ* gene for monitoring the dynamic of the expression changes of the *ACT1* gene (encoding actin, the marker of dividing cells) and *SSA3* gene (encoding a chaperone of the *HSP70* family, the marker of stationary cells) during the approximately 8 days of colony growth. They identified two growth phases in colony development and determined populations of dividing and nondividing cells in particular areas of a colony. In this study, we used the stable version of *lacZ* with the aim of marking all cells within a colony which produced β-galactosidase, regardless of whether the production was stable or transient during the colony development. This allowed us to distinguish cells which never expressed *lacZ* and to monitor *lacZ* switching on in cell progenies during colony growth. In our arrangement, chromogenic X-gal was directly present in the plates, and we suppose that β-galactosidase, once produced, remains within cells long enough to enable the monitoring of the pattern of *lacZ*-expressing and nonexpressing cells during the whole interval of the colony development. However, we are not able to identify eventual *lacZ* repression in cells which previously produced β-galactosidase. It will be interesting to perform more dynamic studies with identified genes using the nonstable version of *lacZ* gene.

In order to increase the sensitivity of β-galactosidase detection, we used chloroform vapors for permeabilization of the cells within a colony in different stages of development. The application of this agent usually led to intensification of the observed blue/white pattern. This result also confirmed that the blue/white coloring is the real result of different production of β-galactosidase in different areas of the colony and not only the result of changed permeability of the cells (e.g., because of the different ages in respective colony areas).

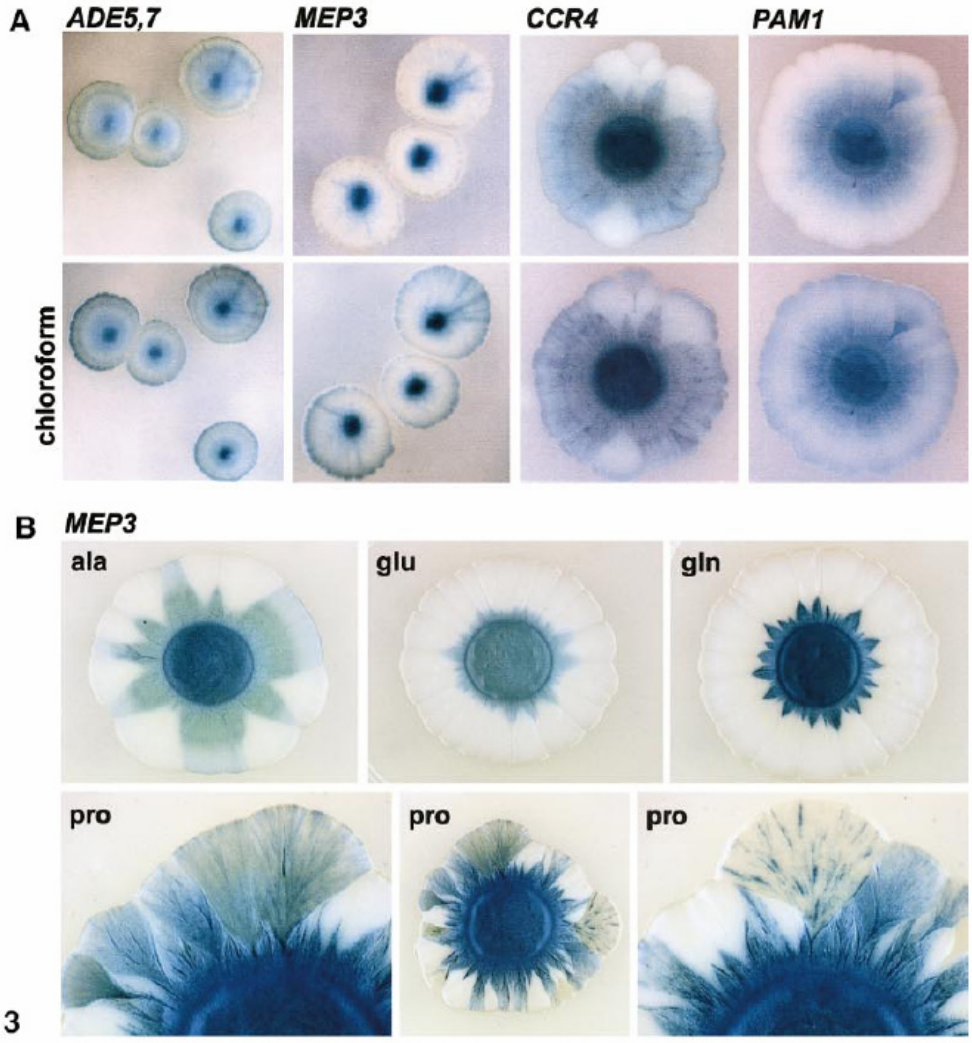
Plasmids containing *lacZ* fusions can be directly used for analyses of activity of the respective promoter in colonies growing under various external conditions. This can help to identify genes which may be involved in regulations and also to find out factors specifically affecting the development of yeast colonies. Here, we followed expression changes of *MEP3-lacZ* fusion on media with different amino acids as sole nitrogen sources. *MEP3* promoter was shown to be under the control of nitrogen repression (repressed by good nitrogen sources as ammonium and glutamine) [16]. The expression of *MEP3-lacZ* in colonies growing on 15 mM ammonium, 10 mM glutamine, or 10 mM glutamic acid was evident mostly in central parts of colonies, whereas the presence of proline (a poor nitrogen source) led to a well-developed expression pattern for the whole colony (Fig. 3B). Monitoring the expression of genes regulated by nutrients thus might also provide indications about particular nutrient "conditions" in different colony areas.

The main limitation of the β-galactosidase/X-gal assay is the dependence of β-galactosidase activity on pH (around neutral [22]). This restricts the versatility of the β-galactosidase/X-gal assay and excludes the possibility of using this approach to study the effects of external pH changes on colony development, which might be, according to our previous observations, an important developmental factor [7, 8]. One possibility for extending the detection possibilities is to fuse genes isolated from the library with the gene for *GFP* (green fluorescent protein) and to set up (if possible) *GFP* detection in colonies. The relevant experiments are under way.

Colonies formed by purified transformants exhibited morphotypes of two basic patterns (or their combinations): (1) Colonies with sectors where individual sectors can have their own specific pattern of β-galactosidase expression; and (2) colonies where the blue/white color is changed in circles. The sectors seem to be formed by clones of the cells, which either switched on (blue sectors) or switched off (white sectors) the expression of the *lacZ* from the respective promoter in different stages of colony development. On the other hand, the appearance of blue and white central rings indicates a synchronization of activity of the promoters in

FIG. 3. (A) The strengthened coloring of the colonies after chloroform treatment. (B) Blue/white color pattern of giant colonies on media with different sole nitrogen sources. (A) Monocolonies of *ADE5,7* and *MEP3* transformants or giant colonies of *CCR4* and *PAM1* were grown on selection MMX medium for 30 days; then volatile chloroform was applied for 60 min, and colonies were incubated for other 24 h. Magnification 1.5× (monocolonies) and 2× (giant colonies). (B) Colonies of *MEP3* transformants were grown on MMX-ala, MMX-glu, MMX-gln, and MMX-pro media for 30 days; then volatile chloroform was applied for 60 min, and colonies were incubated for another 24 h. Magnification 2×.

FIG. 4. Colony pattern formation in the population of the neighboring colonies. Monocolonies of *ADE5,7, PAM1, CAT2, MEP3,* and *CCR4* were grown on MMX medium for 20 days. Magnification 1.2×.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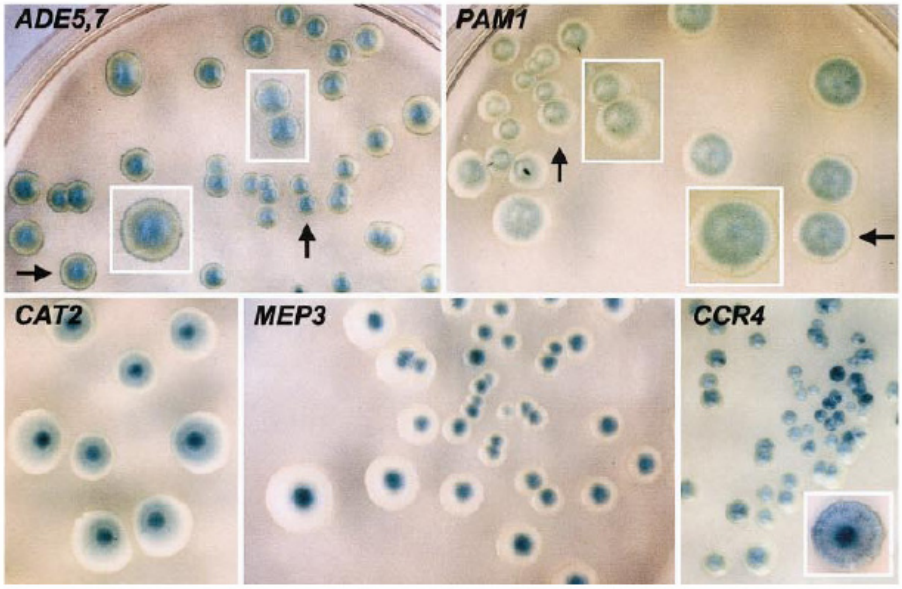
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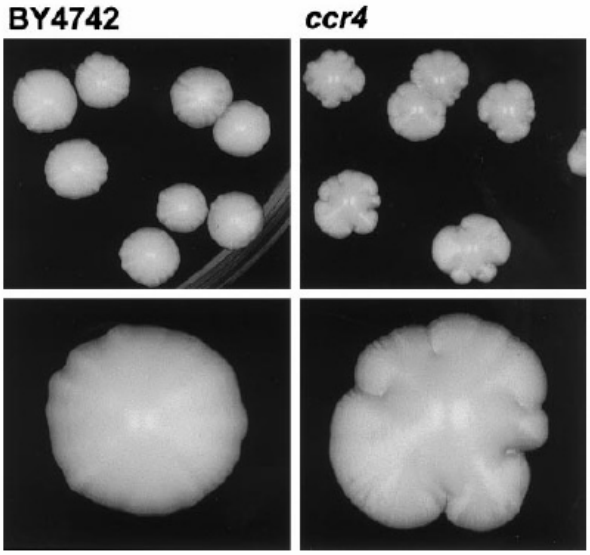

FIG. 5. Morphologies of monocolonies of *S. cerevisiae* BY4247 and *ccr4* mutant. Colonies of BY4742 and isogenic *ccr4* mutant were grown on YEPG medium for 12 days. Magnification 1× and 3×.

cells, which do not originate from one ancestor but have a similar position in the colony. In contrast to monocolonies, the expression patterns in individual giant colonies carrying the same promoter were more variable and more easily influenced by external conditions (medium). Also, the frequency of sector appearance was significantly higher than in monocolonies. Two basic types of sectors could be recognized: those with the staining pattern simulating the pattern of the parental colony and those that stained differently (data not shown). The majority of sectors of giant colonies originated from the central area, where the drop with cell suspension was inoculated, suggesting that the ancestor cell of the clone forming a sector was already present in the cell suspension used for the inoculation. This is in agreement with the observation of rare sector appearance in monocolonies, which usually each arise from one cell only. The appearance of sectors originating in later phases of colony development and reflecting the differentiation of the cells during the colony growth was more rare and appeared when stress conditions (poor nutrient sources) were established (Fig. 3B). Our pattern observations are in agreement with the results obtained with colonies formed by the *E. coli*–containing bacteriophage Mu-*acZ* insertions in different sites of the genome [23–25]. They also exhibited patterned blue/white morphotype, again with two basic patterns (sectors and rings).

When the development of monocolonies formed by individual transformants inoculated in various densities was followed, we observed similar colony coloring in colonies of different sizes. The colonies of *ADE5,7* transformants of the same age (Fig. 4), when present in higher densities, are smaller than those occurring in area with a lower colony concentration. Nevertheless, both the smaller and larger colonies exhibit the same profile of blue and white central rings. This observation indicates either synchronization of *ADE5,7* gene expression in colonies of different sizes or the existence of a regulatory mechanism dependent on colony age (not on colony size). A similar situation was observed when the colony development of *CAT2* and *MEP3* transformants (Fig. 4) was followed, except the diameter of central stained areas was similar in colonies of different size. This might indicate that this central region was formed before the colonies started to differentiate in their size. We previously described the synchronization of a colony development achieved by NH_3 induction [8]. Our present results suggest that the temporal synchronization of the growth of neighboring colonies might be more universal and also could be manifested by the synchronous regulation of expression of particular genes.

The differences in coloring of more dense (smaller) and less dense (larger) colonies of *PAM1-lacZ* and *CCR4-lacZ* transformants (Fig. 4) suggest the existence of an internal mechanism regulating expression of some genes in individual colony, may be with dependence on its topical size. The observation that the regular pattern of *CCR4* expression did not appear on colonies which had no space to grow to a larger size (Fig. 4) indicates that some of the regulatory mechanisms involved in colony pattern formation might be switched on in the colony after it has reached a certain size. This, together with the finding that colonies of *ccr4* mutant exhibit changed, more irregular morphology, indicates that the Ccr4 transcription factor might be involved in regulation at later stages of the colony development. The appearance of additional staining at the central top of larger *PAM1* colonies (Fig. 4) indicates that some genes might be switched on at later stages of the development in the cells, which were probably not dividing. This later increase of the blue color intensity was not a result of more efficient cell permeabilization (due to cell aging) because the chloroform application on small *PAM1* colonies did not lead to the new coloring appearance (data not shown).

All these observations support the general conclusion that colonies formed by microorganisms are organised structures that exhibit differentiated expression of various genes during their development. So far, mainly colonies of *Candida albicans* that exhibit expressive dimorphic transitions and structured colony morphology have been studied [3]. In contrast to *C. albicans,* a bird's view of colonies of *S. cerevisiae* laboratory strains does not reveal a sophisticated, structured colony morphology. Nevertheless, when analyzed by means of *lacZ* fusion plasmids, even these seemingly unstructured colonies displayed specific and characteristic

color pattern reflecting differentiated activities of certain promoters. These results suggest that nonmotile yeast cells differentiate in gene expression within a colony, thus resembling the differentiated gene expression in cells forming tissues of multicellular organisms.

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Aminokyseliny ovlivňují pulzy amoniaku v kvasinkových koloniích.

Amino acids control ammonia pulses in yeast colonies (ZIKANOVA *et al.* 2002).

Kvasinkové kolonie periodicky produkují plynný amoniak, který funguje jako signální molekula působící na dlouhé vzdálenosti. Přijetí takového signálu sousední kolonií indukuje další produkci amoniaku. V této práci jsme se zabývali vlivem amonných iontů a aminokyselin přítomných v médiu na tento proces.

Zjistili jsme, že produkce amoniaku není závislá ani na přítomnosti externího zdroje amonných iontů ani na funkčnosti jejich přenašečů Mep1, Mep2 a Mep3. Komponenty signální dráhy Mep2-Gpa2 rovněž nemají vliv na amoniakové pulzy, jak ukázaly pokusy s různými alelami genu *GPA2*. Sníženou produkci amoniaku však vykazovaly kmeny s deletovanou permeázou aminokyselin Gap1 stejně jako kmeny s defektními senzory hladiny aminokyselin Ssy1 a Ptr3.

Abychom zjistili které aminokyseliny jsou důležité pro amoniakovou signalizaci, testovali jsme produkci amoniaku u kolonií *Candida mogii* rostoucích na minimálním médiu s přídavkem jednotlivých aminokyselin jako jediným zdrojem dusíku. Zjistili jsme, že některé aminokyseliny mají schopnost indukovat produkci amoniaku (např. prolin). Zajímavé je, že morfologie kolonií je závislá na aminokyselině sloužící jako zdroj dusíku. Kolonie rostoucí na médiu s "neindukujícími" aminokyselinami mají vrásčitou morfologii a jsou tvořeny pseudohyfami a hyfami. Naopak kolonie rostoucí na médiu s "indukujícími" aminokyselinami jsou tvořeny kvasinkovými buňkami a jsou poměrně hladké. Indukce produkce amoniaku ovlivňuje jak samotnou morfologii buněk tak i morfologii kolonií. Kolonie rostoucí na médiu s "indukujícími" aminokyselinami udržují mezi sebou určitou vzdálenost, kolonie na médiu s "neindukujícími" aminokyselinami se navzájem přerůstají. Všechna tato pozorování svědčí o vysoce kooperativním chování buněk v kvasinkových koloniích.

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Amino acids control ammonia pulses in yeast colonies

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Abstract

Individual yeast colonies produce pulses of volatile ammonia separated by phases of medium acidification. Colonies of *Saccharomyces cerevisiae* mutant defective in the general amino acid permease, Gap1p, exhibit decreased ammonia production. Mutations in the *S. cerevisiae* amino acid sensor SPS completely abolish the colony ammonia pulses. In contrast, the ammonia pulse production is independent of external concentrations of ammonium and of its uptake by the ammonium permeases Mep1p, Mep2p, and Mep3p. It is concluded that in *S. cerevisiae* colonies, the extracellular amino acids, but not the extracellular ammonium, serve as a source for volatile ammonia production. These phenomena are not restricted to *S. cerevisiae*, since we observe that extracellular levels of 8 out of the 20 tested amino acids are necessary for ammonia pulses produced by *Candida mogii* colonies. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Yeast colonies; Ammonia signalling; Amino acid permeases; SPS sensor; Ammonium permeases

Yeast colonies produce periodically volatile ammonia (NH_3) that acts as a signal between colonies. The first ammonia pulse, starting early after colony inoculation, does not seem to influence neighbouring colonies and is followed quickly by acidification of the agar. The second ammonia pulse enhances ammonia production in neighbouring colonies. This behaviour is important for the spatial distribution of colonies as each ammonia pulse is accompanied by transient inhibition of the growth of facing borders of neighbouring colonies. Thus colonies grow preferentially to the opposite side which is free of competitive colonies [1]. The volatile ammonia produced by a given colony induces ammonia production in neighbouring colonies, regardless of the developmental phase of these. This results in synchronous growth and synchronous ammonia pulses in colony populations [2]. These observations, together with the finding that ammonia induction in colonies of *Candida mogii* is accompanied by spectacular cellular and colony morphology changes [2], support the previously postulated signalling function of volatile ammonia.

What is the source of ammonia produced by colonies? Is it the external ammonium (NH_4^+), transported into the cells or is it the intracellular ammonium originating from amino acid catabolism, which is converted to ammonia (NH_3) and released from colonies? In the latter case one would expect the external amino acids taken up by the cell to be the ultimate origin of the ammonia production.

Three plasma membrane permeases Mep1p, Mep2p, and Mep3p [3] were identified as specific ammonium transporters in *Saccharomyces cerevisiae*. They exhibit significant amino acid sequence homologies between each other and differ in their affinities for NH_4^+ [3]. They are classified as members of the TC 2.A.49 Amt family (http://tcdb.ucsd.edu/tcdb/background.php). The Mep2p permease, besides its ability to transport ammonium, functions also as a sensor for the external ammonium concentration during the transition of *S. cerevisiae* from yeast-like to pseudohyphal growth [4]. Gpa2p, the α-subunit of a G-protein, is functionally associated to Mep2p [5].

Amino acids can be transported into yeast cells by a large phylogenetic family of amino acid permeases. This family, classified as 2.A.3.10 YAT family in the TC nomenclature (http://tcdb.ucsd.edu/tcdb/background.php), comprises the low affinity general amino acid

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permease, Gap1p (which transports the majority of amino acids with high capacity) and several high affinity but low capacity amino acid permeases, which are specific for individual amino acids [6]. The Ssy1p member of this family does not function as a transporter. It plays, together with the two peripherally associated plasma membrane proteins, Ptr3p and Ssy5p, a role of a sensor for extracellular and/or intracellular amino acids (termed as the SPS amino acid sensor) [7]. Ssy1p has a unique 200-amino-acid N-terminal extension that is required for sensor activity [7,8]. In response to extracellular (or intracellular) amino acids, the SPS sensor initiates intracellular signals regulating the functional expression of several amino acid-metabolising enzymes and amino acid permeases [9].

Colonies of the *S. cerevisiae shr3* mutant strain, defective in the subcellular trafficking of various amino acid permeases, as well as colonies of *S. cerevisiae* and *C. mogii* growing in the absence of external amino acids fail to produce ammonia pulses. This indicates a role of the uptake of external amino acids in ammonia signalling [1].

In this paper, we analyse the role of individual amino acids and of different members of amino acid and ammonium transporters in the production of ammonia by yeast colonies.

Table 1
Yeast strains and plasmids used in this study

Strain	Defective	Isogenic	Reference
PLY1		S288C	[10]
HKY37	*ssy1*	PLY1	[8]
HKY38	*ptr3*	PLY1	[8]
PLY126		S288C	[8]
HKY20	*ssy1*	PLY126	[8]
HKY31	*ptr3*	PLY126	[8]
Σ1278b			[11]
Σ1278b	*gap1*	Σ1278b	[12]
HS100-3C	*gap1, lyp1, can1*	Σ1278b	[13]
AM8-1	*can1, lyp1, gap1, shr3*	Σ1278b	[14]
MLY40		Σ1278b	[5]
MLY104	*mep1*	MLY40	[4]
MLY108	*mep2*	MLY40	[4]
MLY128	*mep3*	MLY40	[4]
MLY115	*mep1*, *mep2*	MLY40	[4]
MLY129	*mep1*, *mep3*	MLY40	[4]
MLY130	*mep2*, *mep3*	MLY40	[4]
MLY131	*mep1*, *mep2*, *mep3*	MLY40	[4]
Plasmid	GPA2 allele	Derived	Reference
pML180	GPA2 (wt)	pSEYC68	[5]
pML160	GPA2-2, Gly132Val (activated)	pSEYC68	[5]
pML179	GPA2-3, Gly299Ala (negative)	pSEYC68	[5]

Materials and methods

Strains, plasmids, and media. *Candida mogii* and *S. cerevisiae* OL1 are from the Collection of Yeast Cultures of the Department of Genetics and Microbiology (DMUP), Charles University, Prague. *Saccharomyces cerevisiae* defective in Mep permeases were kindly provided by Lorenz (Duke University, Durham, USA) [4], *ssy1* and *ptr3* strains by Forsberg (Karolinska Institute, Stockholm, Sweden) [7] and *gap1*, *lyp1*, *can1* strains by Sychrova (IF AVCR, Prague, Czech Republic) [12,13]. Plasmids containing *GPA2* alleles are from Lorenz [5]. Detailed list of the strains and plasmids used in this study is in Table 1. *Saccharomyces cerevisiae* OL1 strain was transformed by plasmids with three different allels of *GPA2* gene under the *GAL1-10* promotor (Table 1).

Colonies were grown on GM agar (1% yeast extract, 3% glycerol, 2% agar, 30 mM $CaCl_2$), GM-BKP agar (GM, 0.01% bromcresol purple), GM-BKP + $(NH_4)_2SO_4$ agar (GM-BKP with 5, 20, 50, and 100 mM $(NH_4)_2SO_4$, respectively), MM agar (2% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.1% Wickerham's vitamin solution [15], 2% agar, 30 mM $CaCl_2$), MM-BKP agar (MM, 0.01% bromcresol purple), MM-BKP + $(NH_4)_2SO_4$ agar (MM-BKP with 5, 20, 50, and 100 mM $(NH_4)_2SO_4$, respectively), MM or MM-BKP agar supplemented with 0.5% casamino acids, MM or MM-BKP agar supplemented with all amino acids (1 mM each), MM or MM-BKP agar supplemented with individual amino acids (10 mM each).

Ammonia production measurement. Ammonia released by growing colonies was absorbed into acidic traps as described ([1]; www.natur.cuni.cz/~zdenap/protocols.html) at the intervals indicated in Figs. 1 and 2. The amount of ammonia in various liquid samples was determined by using of the Nessler reagent.

Monitoring of pH pulses during colony development. Colonies of individual strains were grown on GM-BKP agar containing pH indicator bromcresol purple (changing colour from yellow at pH 5.2 to violet at pH 6.8). OL1/*GPA2* transformants were grown on GM-BKP agar with and without galactose.

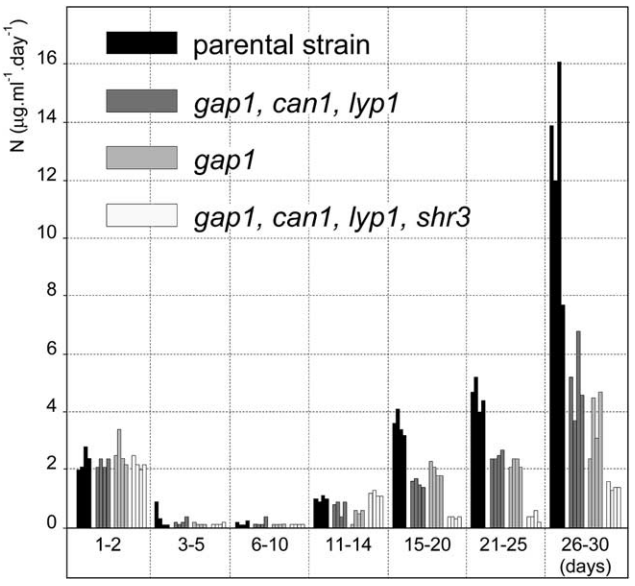

Fig. 1. Production of ammonia by colonies of *S. cerevisiae* defective in amino acid transport. The ammonia produced by colonies growing on GM agar was absorbed (during indicated intervals) and measured as described.

Photography. Colonies were photographed with above illuminating light. A colour camera HITACHI HVC20 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. Cells were photographed using Olympus microscope BX60.

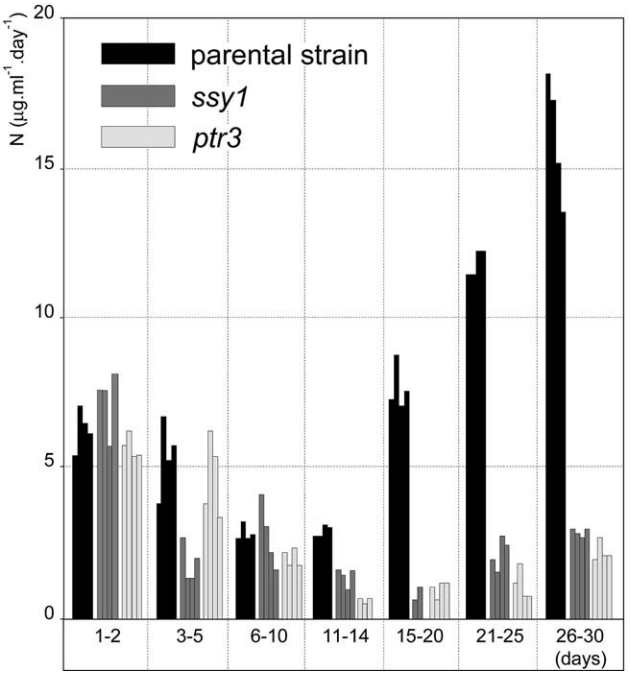

Fig. 2. Production of ammonia by colonies of *S. cerevisiae* defective in amino acid sensors. The ammonia produced by colonies growing on GM agar was absorbed (during indicated intervals) and measured as described.

Results

Ammonia production in colonies of S. cerevisiae and C. mogii is independent of external ammonium and its uptake

To answer the question, whether external ammonium (NH_4^+) influences the production of NH_3, we grew *S. cerevisiae* BY4742 and *C. mogii* colonies on GM-BKP or MM agars with concentrations ranging from 0 to 100 mM of $(NH_4)_2SO_4$ (see Materials and methods). Because of NH_3 evaporation from agar plates containing $(NH_4)_2SO_4$ concentration higher than 20 mM, direct measurement of volatile ammonia produced by colonies was disabled. Therefore, we used a pH dye indicator detecting the alkalisation of the medium around colonies for monitoring the ammonia pulses. We found that the times of ammonia pulse appearance were equal for all tested $(NH_4)_2SO_4$ concentrations. Intensity of alkalisation was even slightly stronger on low ammonium media, which might be caused by buffer capacity differences of the agars (data not shown).

We further investigated the ammonia production by colonies defective in all combinations of Mep ammonium transporters (Table 1). Direct measurement of ammonia production by colonies growing on GM agar was performed in parallel with observations of pH changes around colonies. No significant differences were found as compared to isogenic parental strain (data not shown). In addition, *S. cerevisiae* strains expressing either the activated ($GPA2^{Gly132Val}$) or the dominant negative allele ($GPA2^{Gly299Ala}$) (Table 1) do not exhibit any difference in ammonia pulses when compared to strains expressing the wild *GPA2* allele or containing empty plasmid (data not shown). Thus, neither the transport nor the sensing of the external ammonium seems to be important in ammonia signalling.

Decreased ammonia production in S. cerevisiae colonies defective either in the Gap1p amino acid permease or in the Ssy1p and Ptr3p amino acid sensors

We measured production of ammonia by colonies of *S. cerevisiae* defective in the amino acid permeases Gap1p (general amino acid permease), Lyp1p (specific permease for lysine), and Can1p (specific permease for arginine) (Table 1). Ammonia production in colonies of the *gap1* mutant was significantly reduced at the time of the second pulse when compared with that of isogenic parental strains (Fig. 1). The mutations *lyp1* and *can1* had no additional effect on ammonia production. In agreement with our previous observation [1], the strain *gap1*, *lyp1*, *can1*, containing an additional mutation in the *SHR3* gene completely failed to produce ammonia at the time of the second pulse (Fig. 1).

Measurements of ammonia production in colonies defective in the sensors Ssy1p and Ptr3p (Table 1), growing on complex GM agar revealed that neither *ssy1* nor *ptr3* mutants are able to produce ammonia in the time of the second pulse (Fig. 2). The first pulse ammonia production was not affected in colonies of any mutated strain (Fig. 2) as also observed in colonies of *shr3* mutants [1]. This indicates that the first ammonia pulse is either a consequence of the new protein turnover starting after cell inoculation, or is released during a catabolism of amino acids from preexisting vacuolar stocks.

Ammonia production and morphology of C. mogii colonies are influenced by the presence of certain amino acids

To determine the ability of individual amino acids to sustain ammonia production, we measured ammonia production of giant colonies of *C. mogii* growing on either minimal agar (MM) supplemented with casamino acids (0.5%), or on MM supplemented with the mixture of all 20 amino acids (1 mM each), and also on MM supplemented with individual amino acids (10 mM each). As a control, the ammonia production by colonies growing on MM without any supplement was measured. The results showed two groups of amino acids (Fig. 3B). The first group includes the amino acids Asn, Pro, Asp, Gly, Arg, Glu, Ser, and Ala. The presence of any one of these amino acids induced *C. mogii* colonies to produce ammonia at the time of the second

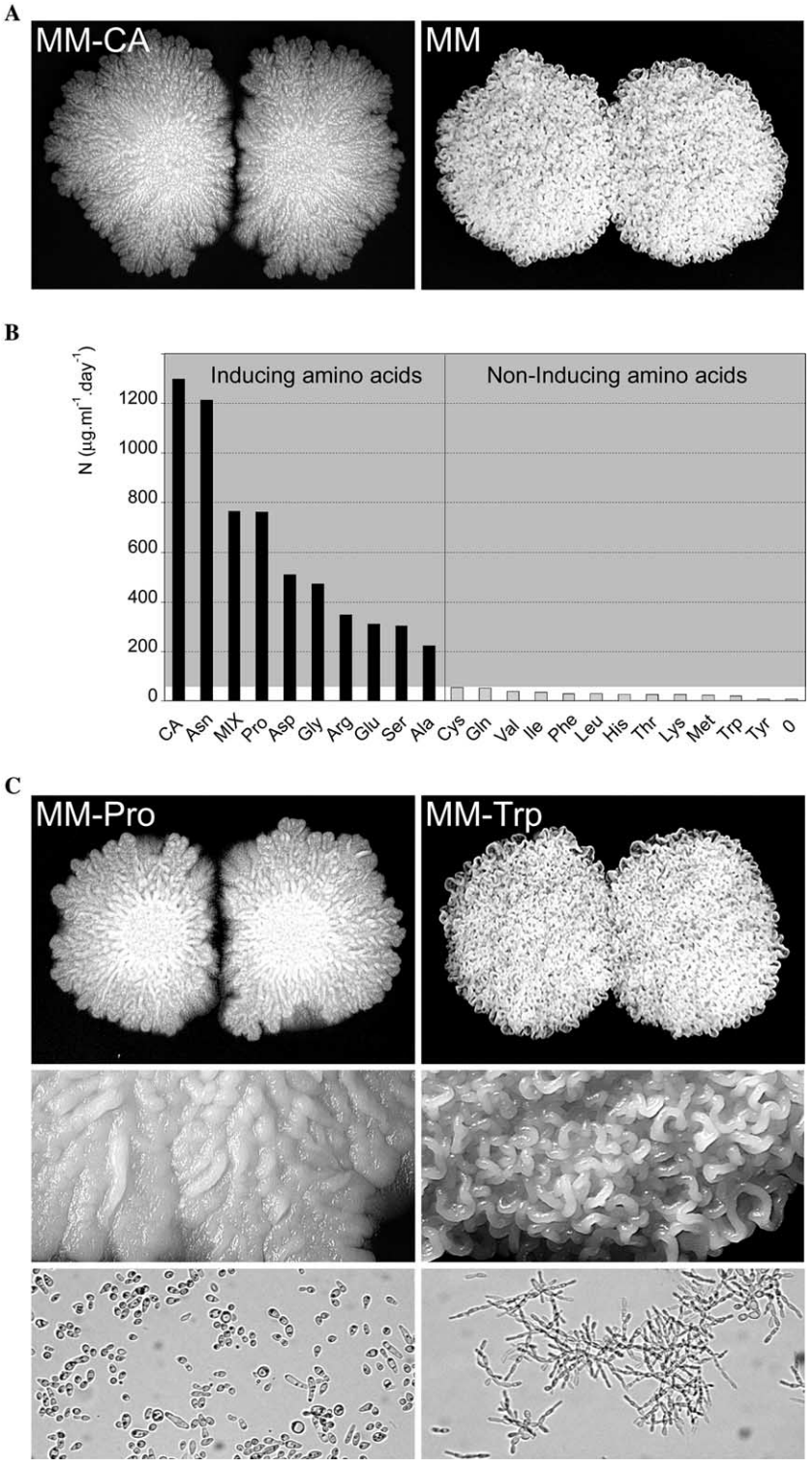

Fig. 3. The presence of certain amino acids in MM agar enables ammonia production. (A, C) Morphologies of *Candida mogii* colonies and cells growing on MM agar supplemented with indicated amino acids. (B) Total amount of ammonia produced during the period of the second pulse by a *C. mogii* colony growing on MM agar supplemented with indicated amino acids. MM-CA (MM agar supplemented with casamino acids) MIX (the mixture of all 20 amino acids). Grey area indicates significant ammonia production.

pulse. In contrast ammonia was detected neither on control MM medium nor on MM supplemented with any other 12 amino acid (Fig. 3B).

The morphologies of *C. mogii* giant colonies growing on MM supplemented with individual amino acids showed two characteristic morphotypes. Rather smooth

colonies similar to those created on MM supplemented with casamino acids were formed on MM supplemented with any inducing amino acid (Figs. 3A and C). In contrast, ruffled colonies grew on most of the non-inducing amino acids (Cys, Val, Phe, Leu, Thr, Met, Trp, Tyr). These ruffled colonies were similar to those growing on pure MM without any supplement (Figs. 3A and C). In agreement with our previous findings [1], ammonia producing colonies growing on inducing amino acids kept a significant distance between themselves, while colonies failing to produce ammonia overgrew each other, specially in the later developmental stages (Fig. 3C). The two types of colonies differed also in the cell composition. Colonies growing on inducing amino acids were formed predominantly by yeast-like cells during ammonia production, while the others were composed by pseudohyphae and hyphae during all tested phases (Fig. 3C).

Besides these two major morphological groups, the presence of Gln, Ile, His, and Lys in the agar resulted in "intermediate" colonies that exhibited morphology rather similar to the smooth morphology of colonies growing on MM supplemented with casamino acids. However, their mutual growth inhibition was less efficient. At the time of full ammonia production, these colonies were formed by a mixture of yeast-shape and hyphae cells. These observations indicate that traces of ammonia, below our detection limit, might be produced by colonies growing on these amino acids.

Discussion

Our results indicate that the ammonia production in yeast colonies is not dependent on external ammonium and on its uptake by the ammonium permeases Mep1p, Mep2p, and Mep3p. Neither the high affinity Mep2p permease, functioning as the sensor of external ammonium [4], nor the Mep2p–Gpa2p pathway, involved in *S. cerevisiae* pseudohyphal differentiation [5], seem to interfere with ammonia production.

In contrast, several observations support our earlier prediction of the involvement of external amino acids and of their uptake in ammonia signalling by yeast colonies. Measurements of ammonia production in *C. mogii* colonies growing on agar supplemented with different amino acids show that capability of different amino acids to induce ammonia production differs (Fig. 3B). The catabolic pathway of most of the inducing amino acids (e.g., Pro, Asp, Gly, and Arg) comprises ammonium production steps (see KEGG, http://www.genome.ad.jp/kegg/kegg2.html). Some of the genes encoding amino acid catabolic enzymes catalysing reactions leading to intracellular release of ammonia or ammonium are induced during the transition of *S. cerevisiae* colonies from acidic to ammonia phase, as indicated by microarray analyses [16]. For example, the induced gene, *GCV1*, encodes glycine decarboxylase participating in ammonia release from glycine (EC 2.1.2.10). Analyses of colonies formed by *S. cerevisiae* strains defective in individual enzymes of amino acid catabolism are underway.

In colonies of *C. mogii*, different cellular types such as yeast-like cells, hyphae or pseudohyphae can be observed. As previously shown, ammonia induction affects both the cellular and colony morphology of this yeast [2]. *C. mogii* colonies growing on non-inducing amino acids have the hyphal morphotype. This is in agreement with our previous finding that *C. mogii* colonies occurring in acidic developmental phase are formed predominantly by pseudohyphae and hyphae [2]. The ammonia induction was accompanied by the transformation of hyphae into yeast-like cells [2], similar to the changes observed in *C. mogii* colonies growing on inducing amino acids. Nevertheless, *C. mogii* colonies growing on complex GM agar exhibit a smooth morphology in acidic phase. During the ammonia induction these smooth colonies changed to the ruffled spaghetti-like structure formed by yeast-like cells [2]. In contrast, *C. mogii* colonies growing on minimal agar supplemented with non-inducing amino acids never reached the smooth morphology. Instead they exhibit ruffled morphology formed mostly by hyphae/pseudohyphae throughout their development. These results demonstrate that there is no strict relation between colony morphology and the shape of the cells within the colony.

Efficient uptake of amino acids mediated by the high capacity general amino acid permease Gap1p proves to be important for ammonia production. On the contrary, the contribution of the specific amino acid permeases Can1p or Lyp1p to the intracellular amino acid pool has no significant effect on the amount of released ammonia (Fig. 1). The necessity of the presence and of the uptake of amino acids together with observed changes in expression of amino acid metabolic genes and the transient decrease of intracellular amino acid pool at the beginning of ammonia production [16] indicate that amino acids are the initial source of ammonia in yeast colonies. Nevertheless, the behaviour of *S. cerevisiae* strains, defective in the SPS amino acid sensors, suggests that the role of amino acids in pulse ammonia production is more general than just being the catabolic source of ammonia. It was previously demonstrated that *ssy1* and *ptr3* mutants express higher levels of *GAP1* mRNA when grown on amino acid rich media as YPD [8]. Therefore, Gap1p permease should be present in *ssy1* and *ptr3* colonies and should import amino acids from the surroundings. Despite of that, colonies of both, *ssy1* and *ptr3* mutants produce even less ammonia at the time of the second pulse than the colonies of the *gap1* mutant (compare Figs. 1 and 2). These observations imply that the extracellular and/or intracellular concentration of amino acids might be important for the decision of colonies to switch to the ammonia producing phase and

that the defect in amino acid sensor components affects this decision. Additionally, *ssy1* and *ptr3* mutants exhibit changes in ratios of the distribution of intracellular amino acid pool between vacuole and cytoplasm leading to increased vacuolar pools of several amino acids (e.g., arginine and histidine [8]). The *ssy1* and *ptr3* mutations thus might negatively influence the release of amino acids from vacuoles, which occurs during the acid to alkali colony transition [16].

In brief, our results raise the possibility that in yeast colonies the extracellular amino acids function not only as sources of ammonium but also as indicators for the decision to switch from acidic to the ammonia producing developmental phase thus implicating a possible biological role of SPS amino acid sensor in long-term colony development.

Acknowledgments

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Domestikace divokého kmene *Saccharomyces cerevisiae* je doprovázena změnami genové exprese a morfologie kolonií

Domestication of wild *Saccharomyces cerevisiae* is accompanied by changes in gene expression and colony morphology (KUTHAN *et al.* 2003).

Laboratorní kmeny kvasinky *Saccharomyces cerevisiae* tvoří na pevném médiu málo strukturované kolonie s hladkou morfologií. Naproti tomu mnohé přírodní izoláty *Saccharomyces cerevisiae* tvoří bohatě strukturované kolonie s drsnou morfologií.

Zajímalo nás, čím se odlišují kmeny tvořící strukturované kolonie od laboratorních kmenů s hladkými koloniemi. Tato otázka je zajímavá i z ryze praktického hlediska, protože strukturovaná morfologie kolonií je u patogenních kvasinek rodu *Candida* či *Cryptococcus* spojována s jejich zvýšenou virulencí.

Pomocí environmentální skenovací elektronové mikroskopie jsme studovali kolonie laboratorního kmene a divokého kmene BR-fluffy. Zjistili jsme, že buňky v koloniích divokého kmene BR-fluffy jsou navzájem spojeny extracelulární hmotou, která není přítomna u laboratorního kmene. Extracelulární materiál obsahuje bohatě glykozylovaný protein o velikosti přibližně 200 kDa, který není příbuzný flokulinům odpovědným za buněčnou adhezi v tekutých médiích.

Divoký kmen BR-fluffy nám připravil ještě další zajímavé překvapení. Při jeho delší kultivaci v laboratorních podmínkách se postupně začaly s poměrně vysokou frekvencí objevovat hladké kolonie nerozeznatelné od kolonií laboratorních kmenů. Touto "domestikací" divokého kmene jsme získali ideální experimentální systém pro zjištění odlišností obou morfologických variant. Ukázalo se, že domestikace je spojena se ztrátou extracelulárního materiálu a výraznými změnami genové exprese. Analýza pomocí DNA microarrays ukázala, že dochází ke změnám exprese přibližně 320 genů. Nejvýraznější změny exprese zahrnují metabolizmus a transport sacharidů, syntézu buněčné stěny, buněčný cyklus a polaritu, aquaporiny, Ty-transpozony a subtelomerické geny.

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Domestication of wild *Saccharomyces cerevisiae* is accompanied by changes in gene expression and colony morphology

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Summary

Although colonies from *Saccharomyces cerevisiae* laboratory strains are smooth, those isolated from nature exhibit a structured fluffy pattern. Environmental scanning electron microscopy shows that the cells within wild fluffy colonies are connected by extracellular matrix (ECM) material. This material contains a protein of about 200 kDa unrelated to the flocculins, proteins involved in cell–cell adhesion in liquid media. The matrix material binds to concanavalin A. Within a few passages on rich agar medium, the wild strains switch from the fluffy to the smooth colony morphology. This domestication is accompanied by loss of the ECM and by extensive changes in gene expression as detected by DNA microarrays. The expression of about 320 genes was changed in smooth colonies. The major changes comprise carbohydrate metabolism, cell wall, water channels, Ty-transposons and subtelomeric genes, iron homeostasis, vitamin metabolism and cell cycle and polarity. The growth in fluffy colonies may represent a metabolic strategy for survival of yeast under unfavourable conditions that is switched off under felicitous laboratory conditions.

Introduction

In nature, the formation of multicellular structures, such as biofilms or colonies, by unicellular microorganisms attached to solid surfaces is a predominant but poorly understood phenomenon. Multicellular life helps microorganisms to survive starvation and other deleterious conditions. Biofilms contain well-developed extracellular material making the microbial cells more resistant to unfavourable external conditions (e.g. various antibiotics; Lewis, 2001). This material is also involved in scaffold composition of biofilms creating channels for water and nutrient flow to individual cells (Costerton *et al*., 1995). Colonies of non-conventional yeast species (e.g. *Candida* or *Kluyveromyces*) usually exhibit more structured morphology than colonies of *Saccharomyces cerevisiae* laboratory strains. Scanning electron microscopy of *Candida albicans* colonies revealed various layers of cells exhibiting different morphologies termed yeast-like, pseudohyphae or hyphae. Some of the cells are connected by fibrillar structures (Whittaker and Drucker, 1970; Radford *et al*., 1994). *C. albicans* is capable of undergoing a different type of cell and colony morphological change that has been termed 'phenotypic switching'. Such changes in the organization of colonies connected with differences in adhesive properties and in invasive growth influence the virulence of clinical pathogen *C. albicans* (Staib *et al*., 2001). Varon and Choder (2000) observed the existence of thin fibrils in starving colonies of laboratory *S. cerevisiae* strains only in late stationary phase. Engelberg *et al*. (1998) described conditions enabling quaint multicellular yeast formations reminiscent of the stalks of *Dictyostelium discoideum* and composed of distinct cell layers (Scherz *et al*., 2001).

In contrast to laboratory strains, various vineyard *S. cerevisiae* strains have the capability to form colonies with structured morphology (Cavalieri *et al*., 2000). We used environmental scanning electron microscopy (ESEM) to compare the native ultrastructure of wild fluffy *S. cerevisiae* colonies with that of the smooth laboratory colonies. We observed that cells within fluffy *S. cerevisiae* colonies are connected by an abundant extracellular matrix (ECM), undetected so far, and organized in a structure reminiscent of multicellular tissues. We also show that, when grown under laboratory conditions, the wild strains switch to the smooth colony morphotype indistinguishable from that of laboratory strains. This 'domestication' is accom-

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panied by specific changes in gene expression, as analysed by hybridization analysis on DNA microarrays.

Results

Colonies of wild S. cerevisiae *are able to switch from the fluffy to the smooth morphology*

Colonies of *S. cerevisiae* laboratory strains usually exhibit smooth and poorly structured morphology. In contrast, the wild *S. cerevisiae* strain BR isolated from nature forms well-structured fluffy colonies (Fig. 1) when growing on GMA (complex agar medium with glycerol) or YEGA (complex agar medium with glucose). The pulsed-field gel electrophoretic (PFGE) distribution of chromosomes confirms the affiliation of our BR-fluffy strain to the *Saccharomyces* sp. (data not shown). The BR-fluffy strain is able to sporulate, which indicates its a/α diploid genotype. Its fluffy morphology does not seem to result from pseudohyphal growth of cells within the colony, but rather originates from the arrangement of individual cells in the fluffy structure already visible in small 3-day-old colonies (Fig. 1).

During prolonged cultivation of the BR-fluffy strain on rich agar medium (either GMA or YEGA), smooth colonies resembling the colonies of conventional laboratory strains appeared (Fig. 1). After replating, their cells again form smooth colonies on both agar media tested. As well as the morphology changes, the fluffy-to-smooth switch is accompanied by changes in cell morphology. Elongated cells frequently visible in fluffy colonies are replaced by oval cells in smooth colonies (Fig. 1). The number of cells creating smooth colonies increases within the BR population during passages on rich agar until the whole population switches to the smooth colony morphotype. On average, when one fluffy colony formed by $\approx 10^7$ cells is picked up, diluted and plated on rich agar, about 2–3% of monocolonies exhibit the smooth morphotype. The high efficiency of the fluffy-to-smooth switch implies that it is likely to be caused by a regulation change within the fluffy cells rather than by a mutation. The fluffy-to-smooth switch is not connected to significant chromosome rearrangements, and the smooth cells keep their a/α properties (data not shown).

Abundant ECM connects cells within fluffy colonies

To analyse the surface of 7-day-old BR-fluffy and BR-smooth colonies growing on GM, we developed two different protocols for ESEM sample preparation (see *Experimental procedures*). The structure-preserving protocol revealed that, on the surface of the smooth colony, the cells are in close proximity. In contrast, the surface of the fluffy colony is rough, and groups of cells appear to be separated by holes (Fig. 2A). This observation indicates the presence of channels connecting the internal and external areas of the BR-fluffy colony. The matrix-preserving protocol revealed that cells within the fluffy colonies are covered by an extracellular capsule and connected by an extracellular material, which extends to visible filaments during the microscopy procedure (Fig. 2B). In contrast, such filaments were rarely observed within smooth colonies (Fig. 2B).

To obtain more information on the development of BR-fluffy and BR-smooth colonies, we analysed their development over 9 days using both microscopy protocols. The first detectable extracellular filaments appear in 2-day-old fluffy colonies and become frequent in 3- to 5-day-old fluffy colonies (Fig. 3). Even within 1-day-old fluffy colonies, the cells are stickier, and their three-dimensional colony structure is better developed than that of 1-day-old smooth colonies (Figs 1 and 3). Later, in 9-day-old fluffy colonies, the amount of intercellular material increases, and the colonies start to be covered with a capsule layer (Fig. 3). Neither the surface 'capsule' nor the well-developed intercellular matrix was found in smooth colonies at any time. Individual filaments were observed only occasionally in BR-smooth colonies (Fig. 3). As shown in

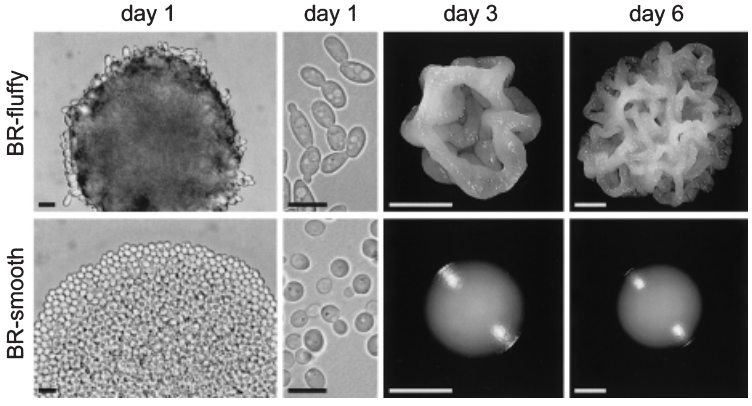

Fig. 1. Comparison of BR-fluffy and BR-smooth colonies growing on GMA medium. Morphology and size of colonies at day 1, day 3 and day 5 of their development. Morphology of cells from 1-day-old fluffy and smooth colonies. Scale: black bars are 10 μm, white bars are 1 mm.

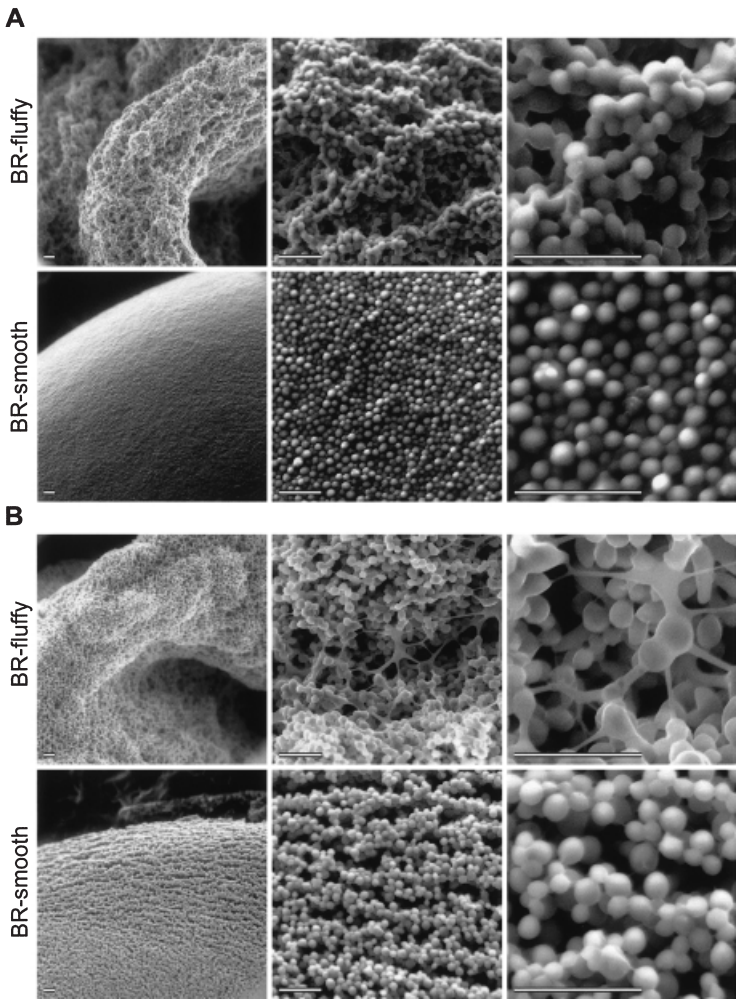

Fig. 2. Ultrastructure of BR-fluffy and BR-smooth 7-day-old colonies growing on GMA medium.
A. Top surface of the colonies observed by ESEM, using structure-preserving protocol.
B. Top surface of the colonies observed by ESEM, using matrix-preserving protocol, which enables visualization of ECM material. All scale bars are 20 μm. The same magnifications were used in (A) and (B).

Fig. 1, from day 3, the BR-fluffy colonies start to be significantly larger than the BR-smooth colonies. This difference results from the cells within BR-fluffy colonies being more dispersed within the colony, rather than from the number of cells forming both types of colonies (data not shown). This feature makes fluffy colonies more competitive for space occupancy.

ECM of the BR-fluffy colonies contains specific compound(s) unrelated to the flocculins

We extracted the extracellular material from 7-day-old fluffy colonies and from smooth colonies of the laboratory strain *S. cerevisiae* GRF18 using sequential extraction with different buffers. The extracts from BR-fluffy colonies contain a major protein of mobility >200 kDa, predominantly in the fraction obtained by washing the cells with PBS buffer without any detergent. This protein appears neither in extracts of GRF18 laboratory colonies nor in those of BR-smooth colonies (Fig. 4A). This indicates that this high-molecular-weight protein (or protein complex) might be a component of the ECM produced by fluffy colonies. Its extracellular localization and the absence of covalent linkage to the cell wall is apparent from the fact that it can be extracted by simple washing of colonies with PBS buffer. Its sensitivity to proteinase K shows its proteinaceous character (Fig. 4C). Despite the fact that concanavalin A (ConA)-peroxidase staining revealed its glycosylated nature (Fig. 4B), glycosidase H, glycosidase O or PGNase (Fig. 4C) were unable to remove glycosidic groups from the extracted material. Owing to the resistance to the tested glycosidases, it was not possible to characterize either the protein or the polysaccharide moiety further.

The cell wall Flo proteins of *S. cerevisiae* are related to the adhesins of pathogenic fungi (Teunissen and Steensma, 1995; Caro *et al.*, 1997; Lo and Dranginis, 1998). The adhesion mediated by one group of Flo proteins, termed flocculins (encoded by genes *FLO1*, *FLO5*, *FLO9* and *FLO10*) is Ca^{2+} dependent and therefore is

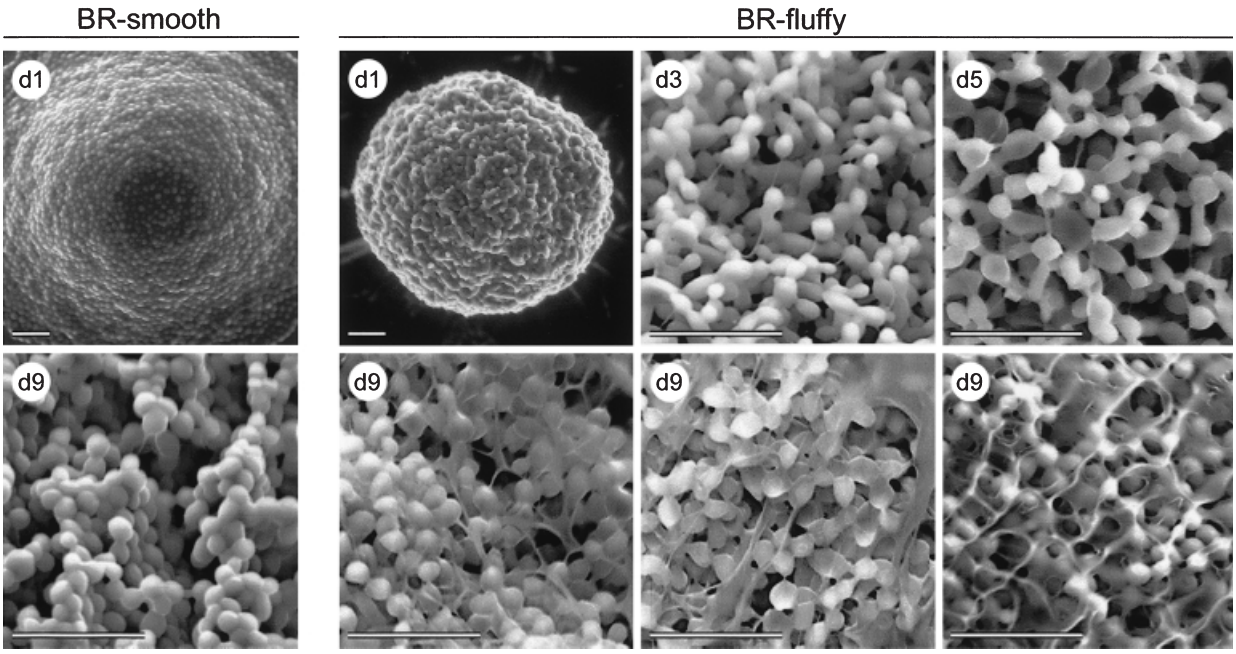

Fig. 3. Ultrastructure of BR-fluffy and BR-smooth colonies growing on GMA medium at different stages of their development (d, age of colonies in days). Top surface of the colonies observed by ESEM using matrix-preserving protocol. Nine-day-old (d9) fluffy colonies are covered by abundant ECM material. All scale bars are 20 μm.

disrupted in citrate buffer. It is also inhibited by the presence of saccharides such as mannose (Stratford *et al*., 1988). Flo11p, a member of the second group of the Flo family, is required for pseudohyphal growth of diploids, for adhesion to the agar surface and for invasive growth of haploids. We grew BR-fluffy, BR-smooth and P23 (well-flocculating brewery strain) as both colonies on complex GMA-Ca or suspensions in the same GM-Ca liquid medium. In parallel experiments, media containing mannose (MnMA-Ca, MnM-Ca) were used. The cells picked up from colonies or centrifuged from liquid culture were suspended in either distilled water or citrate buffer. Results summarized in Table 1 show that the strain BR-smooth does not flocculate in any media. The strain BR-fluffy flocculates in both liquid and solid media. In contrast to the liquid BR cells, the BR-fluffy cells originating from colonies remain in clusters even when suspended in citrate buffer or grown in the presence of mannose. These observations indicate that the ECM responsible for fluffy cell–cell connection is not related to the Flo flocculins and is produced only in the colonies and not in the liquid culture (Table 1).

The flocculation of the strain producing normal levels of Flo11p is also Ca^{2+} dependent and is disrupted by citrate buffer (Lo and Dranginis, 1996). In contrast, Guo *et al*. (2000) reported that aggregation of the Σ1278 strain overexpressing the *FLO11* gene is Ca^{2+} independent and is not inhibited by the presence of mannose. Flo11p is produced at high levels in haploid cells and in filamentous diploid cells, but not in yeast-form diploid cells (Guo *et al*.,

Table 1. Aggregation of BR-fluffy cells taken from colonies is not disrupted by citrate buffer or by the presence of mannose.

	Liquid medium				Colonies on agar			
	GM		MnM		GMA		MnMA	
Strain	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer
P23	+	–	–	ND	+	–	–	ND
BR-fluffy	+	+/–	–	ND	+	+	+	+
BR-smooth	+/–	–	–	ND	+/–	–	–	ND

Individual strains were grown in either liquid media (GM or MnM with mannose) or colonies on the same solid media. Cells were suspended in either water or citrate buffer and observed under the microscope. (+) aggregated cells; (–) individual cells; (+/–) partially aggregated cells; (ND) not detected.

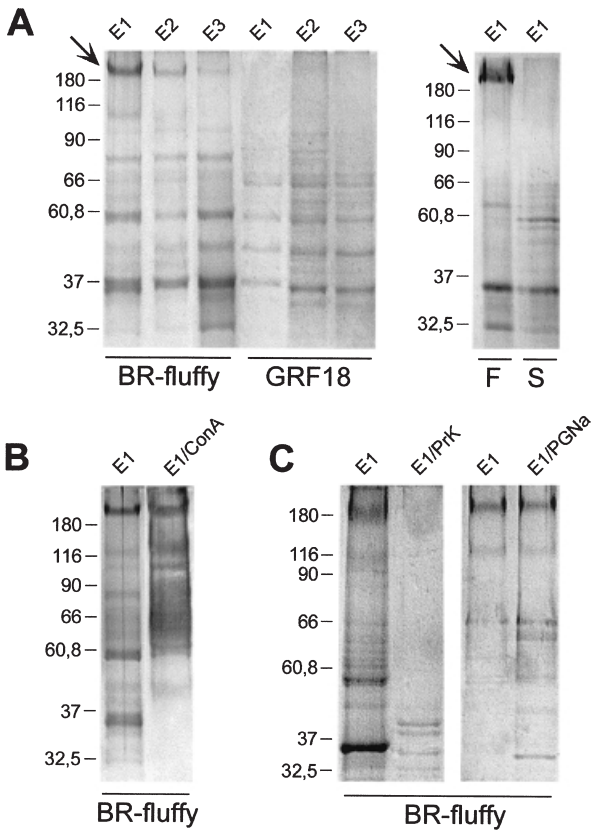

Fig. 4. Extracts from BR-fluffy colonies contain specific protein (indicated by arrow) (A), which is glycosylated (B), sensitive to proteinase K and resistant to PGNase treatment (C). BR-fluffy (F), BR-smooth (S) and GRF18 7-day-old colonies were extracted as indicated in *Experimental procedures*. E1, PBS extract; E2, PBS–Tween extract; E3, PBS–SDS extract; E1/ConA, E1 extract was blotted on membrane, and glycoproteins were visualized as indicated in *Experimental procedures*; E1/PrK, E1 extract was treated with 0.1 mg ml^{-1} proteinase K before loading on PAGE; E1/PGNa, E1 extract was treated with PGNase before loading on PAGE. Marker: M_r ($\times 10^3$).

2000). Also, Flo11p belongs to the group of cell wall glycoproteins predicted to be covalently linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. Fluffy colonies are composed predominantly of yeast-form diploid cells, and the intercellular material can easily be extracted from colonies, excluding its covalent linkage to the cell surface. The *FLO11* signal in microarray comparison of mRNAs isolated from fluffy and smooth colonies (see below) is very low, does not allow any quantification and suggested weak *FLO11* expression. Nevertheless, Northern blot analysis of total RNAs, using the probe specific against the 3′-terminus of *FLO11*, revealed that *FLO11* is more strongly expressed in fluffy colonies compared with the smooth ones (Fig. 5A). On the other hand, the level of expression of Flo flocculins (*FLO1*, *FLO9* and *FLO10*) does not exhibit significant differences (Fig. 5A)

The fluffy to smooth switch is accompanied by gene expression changes

In order to characterize differences between fluffy and smooth colonies at the gene expression level, we performed genome-wide analyses of transcriptomes isolated from both types of BR colonies, each at two different stages of development (4 and 7 days). Significant and reproducible differences among fluffy and smooth transcriptomes were found in the expression of about 320 genes (6% of yeast genes; see complete list in *Supplementary material*). The expression of about 110 genes differs in fluffy and smooth colonies at both developmental stages. Some of the other developmental stage-specific changes might be more related to differences in the timing of fluffy and smooth colony development. The changes in expression of selected genes were confirmed by Northern hybridization (Fig. 5C).

In fluffy colonies, several genes connected with carbohydrate metabolism and transport are significantly upregulated compared with their smooth counterparts (Table 2). They include genes encoding maltases (e.g. *YDL037C*, *MAL12*, *MAL32*, *FSP2*, *YIL172C*), maltose permeases (*MAL31*, *YDL247W*, *YJR160C*), sorbitol dehydrogenases (*SOR1*, *YDL246C*) and genes encoding proteins putatively involved in protein glycosylation (e.g. *YHR210c*, *FBP1*). This is consistent with the observation that ECM produced in fluffy colonies is glycosylated. As the material is resistant against different endoglycosidases, one can infer the existence of cross-linked carbohydrate chains. The gene *AQY1* encoding aquaporin (water channel) is strongly induced in fluffy colonies, thus indicating differ-

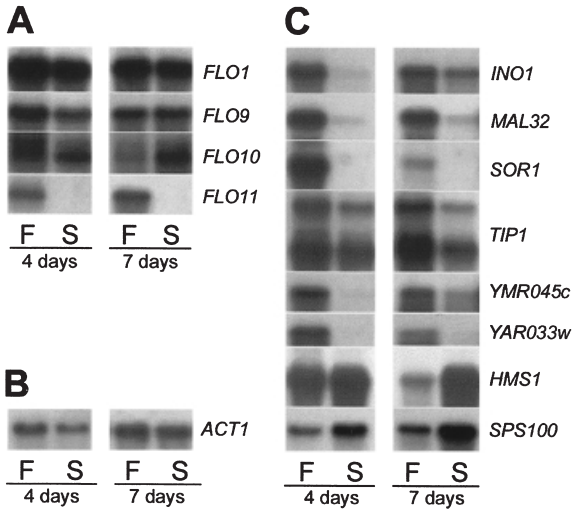

Fig. 5. Northern hybridization of total RNAs isolated from 4-day-old or 7-day-old fluffy (F) and smooth (S) colonies. Probes for flocculin genes and *FLO11* (A), probes for the genes that were induced or repressed in fluffy colonies as indicated by microarray experiment (C) and the probe for the unregulated control, *ACT1* (B) were applied.

Table 2. Differences in gene expression between fluffy and smooth colonies at two different stages of the development of the gene expression level (4 and 7 days)[a].

Genes induced in fluffy colonies	4 days old	7 days old	Genes induced in smooth colonies	4 days old	7 days old
Carbohydrate metabolism/transport			Cell wall		
Glucosidases (maltases and glucanases)			*SPS100*, *CHS1*, *YGP1*, *BGL2*	2.0–4.0	2.0–3.3
MAL12, *MAL32*, *FSP2*, *YIL172C*, *YOL157C*	2.5–7.0	2.4–4.4	*CTS1*, *SSR1*		
YGR287C, *YJL216C*, *YDL037C*			Cell stress/resistance		
Maltose permeases			Aryl-alcohol dehydrogenases		
MAL31, *YDL247W*, *YJR160C*	2.2	2.2–2.9	*AAD3*, *AAD6*, *AAD10*, *AAD14*,	2.0–2.9	2.0–5.0
Sorbitol catabolism			*YFL057C*		
SOR1, *YDL246C*	15.0–21.0	7.0–8.0	Others		
Glycosylation/others			*YGP1*, *CUP1*, *CRS5*, *HSP30*	2.0–2.5	2.0–3.3
YHR210C, *FBP1*	3.1–4.8	4.0–4.4	Iron homeostasis		
Inositol derivatives/vesicular transport			*ENB1*, *NFS1*, *FRE6*, *TIS11*	2.0–3.3	2.5–4.0
INO1, *YJL145W*	2.4–4.4		Transcription regulation		
Cell wall			*HMS1*, *USV1*, *TIS11*	2.2–3.3	2.2–5.0
TIP1	3.9	4.5			
Aquaporins					
AQY1	4.7	3.3			
Iron homeostasis					
FET4, *FIT3*, *FRE5*	2.6–6.6	2.0–4.0			
Transposons					
Ty1-transposons (A and B)[b]					
YAR009C, *YER138C*, *YJR027W*, *YBL005W-B*, *YMR046C*, *YHR214C-B*, *YML039W*, *YMR045C*, *YMR050C*	2.8–4.6	2.0–2.7			
Ty-transposition enhancer					
TEA1	3.0	2.0			
Subtelomeric genes					
YAR027W, *YAR033W*, *YGL051W*	3.6–8.0	2.2–3.6			

a. mRNAs isolated from fluffy colonies (4 or 7 days old) were compared with those isolated from smooth colonies (4 or 7 days old respectively).
b. As all *Ty1B* and all *Ty1A* genes present in the yeast genome are identical in sequence, microarrays cannot distinguish which transposon insertion is activated in fluffy colonies.

ences in water permeability and in surface properties of cells within fluffy and smooth colonies. It has been shown that aquaporin mutants exhibit increased hydrophobicity and, therefore, a higher rate of cell–cell aggregation (Carbrey *et al*., 2001). Increased expression of aquaporin might thus be important for keeping the distances between the cells in fluffy colonies, as observed by ESEM.

Several genes, the products of which are involved in vesicular trafficking (e.g. *SAC2*, *STP22*), are induced predominantly in 4-day-old fluffy colonies (see *Supplementary material*). These genes comprise those involved in inositol regulation of trafficking (*INP53*, *YJL145W*, *YJR110W*) and also in inositol biosynthesis (*INO1*). Their activation suggests enhanced secretion of ECM material within fluffy colonies and indicates regulation by the inositol pathway. One of the most strongly induced genes, *TIP1*, encodes a putative cell wall mannoprotein, possibly connected with cell wall glucan (Kowalski *et al*., 1995). Despite the fact that Tip1p was reported to be covalently attached to the cell wall in laboratory strains (Kowalski *et al*., 1995), we cannot exclude its participation in ECM formation and cell–cell connection in fluffy colonies.

Two other large groups of genes are induced in fluffy colonies in both developmental stages. One group includes several Ty-transposons (e.g. *YER138C*, *YJR027W*, *YMR046C* and *YAR009C*) and the *TEA1* gene encoding a transcription regulator, which binds to the Ty enhancer. The second group includes several subtelomeric genes with unknown function (e.g. *YAR027W*, *YAR033W* and *YGL051W*). Transposon overexpression has been related to stress responses (Bradshaw and McEntee, 1989). However, several genes connected with the general stress response are downregulated in the fluffy colonies compared with the smooth ones (Table 2). Both subtelomeric region expression levels and Ty transpositions might be affected by differences in chromatin structure (Rinckel and Garfinkel, 1996). Moreover, Ty elements are proposed to play a role in the establishment of methylation patterns in plants and maybe in filamentous fungi (for a review, see Martienssen and Colot, 2001). Interestingly, the activation of *Ty1* transcription and retrotransposition have also been shown to be controlled by environmental signals that affect cell differentiation in yeast (Morillon *et al*., 2000). Therefore, the induction of transposons and subtelomeric genes might result from global changes in chromatin structure in fluffy cells and might be relevant to the observed changes in colony structure and cell behaviour as a response to topical environmental conditions. The fact that some of the genes encoding proteins involved in chromatin modification

(*SAS3*, *HPA2*) are induced in 4-day-old fluffy colonies (see *Supplementary material*) supports this possibility.

The pattern of expression of several genes involved in iron homeostasis (e.g. *FET4*, *FIT3*, *FRE5*) differs greatly between the two types of colonies. It is worthy of note that iron availability and uptake have been shown to influence the cell wall composition and adhesion properties of *C. albicans* cells. Despite the fact that the mechanism is unknown, metallic ions might have a significant role in the morphology differences of *S. cerevisiae* colonies.

Several genes encoding transcription regulators (e.g. *IME1*, *MGA1*, *CAF17*) and proteins involved in signalling (e.g. *RCK1*, *REG2*) are induced in fluffy colonies, whereas some of the other transcription factor genes are repressed (e.g. *HMS1*, *USV1* and *TIS11*). Transcription factor Caf17p (induced strongly in 4-day-old fluffy colonies; see *Supplementary material*) is a component of the Ccr4p transcription complex. In previous experiments, we have shown that *CCR4* expression varies during colony development and that the strains with *ccr4* deletion exhibit changes in colony morphology (Minarikova *et al*., 2001).

Some differences occur predominantly in the early developmental stages (4-day-old colonies; see *Supplementary material*). For instance, a group of genes involved in cell division and polarity (e.g. *MSB1*, *MSB2*, *GIN4*, *BFA1*, *ARP10*) is induced in the young fluffy colonies. This indicates that cells are dividing more actively and/or that the polarity of the division is important for the organization of cells within young fluffy colonies. Some of these genes might also participate in morphology differences of cells forming fluffy and smooth colonies (Fig. 1). Likewise, several genes involved in vitamin (biotin, pyridoxine, thiamine and nicotinic acid) metabolism (e.g. *BIO2*, *BIO3*, *BIO4*, *BIO5*, *SNZ1*, *SNZ2*, *SNZ3*, *SNO1*, *SNO2*, *THI4*, *THI7*) are strongly induced in young fluffy colonies. This suggests that the growth of fluffy colonies is more dependent on these components than the growth of the smooth, domesticated ones.

Discussion

The use of two recently available technologies, environmental scanning electron microscopy (ESEM), allowing the preservation and observation of native cellular structures, and DNA microarray hybridization, allowing genome-wide expression analyses, has provided a novel insight into the morphological and metabolic processes accompanying the domestication of wild *S. cerevisiae* into laboratory strains. For the first time, ESEM revealed the detailed native ultrastructure of fluffy *S. cerevisiae* colonies isolated from nature. We have shown that the cells within wild colonies secrete an abundant and specific ECM allowing them to form highly structured and organized colonies. The presence of intercellular filaments even in young fluffy colonies indicates that the production of ECM covering and connecting the cells is not a consequence of colony starvation. Therefore, this phenomenon is different from that observed by Varon and Choder (2000) in starving laboratory *S. cerevisiae* colonies. The capsular ECM in fluffy colonies might be important for keeping the distances between the cells and for protecting the whole colony against unpleasant conditions prevalent in nature. Preliminary observation of five other *S. cerevisiae* BR strains isolated independently from various natural environments indicates that the fluffy colony morphotype, the presence of ECM material and domestication under laboratory conditions are general features of wild *S. cerevisiae* isolates.

The properties of the ECM differ from those of flocculins (Flo1p, Flo5p, Flo9p and Flo10p), proteins responsible for cell–cell adhesion in liquid cultures (Table 1). Available data regarding another *S. cerevisiae* adhesin, Flo11p, are rather inconsistent and do not allow perspicuous conclusions (see also above). FLO11 is expressed more strongly in fluffy colonies compared with the smooth ones (Fig. 5A). Nevertheless, other authors overproducing Flo11p in diploid laboratory strains observed conspicuous changes in strain invasiveness; however, they did not notice any effect of Flo11p overproduction on colony morphology (Lo and Dranginis, 1998; Guo *et al*., 2000). Meanwhile, Flo11p might be involved in the linkage of cells with a fluffy colony matrix formed by some as yet uncharacterized cell–cell adhesion component. Biochemical and microarray analyses gave some information on the nature of the ECM. Unfortunately, its resistance to endoglycosidases makes mass spectrometry analysis difficult. As for bacterial surface glycoproteins, resistance against endoglycosidases indicates the existence of cross-linked carbohydrate chains that might form the extracellular capsule in the fluffy yeast colonies. In fluffy colonies, cells are separated by channels, which have been shown to be important for exchanges of nutrients and waste products in microbial biofilms (for a review, see Costerton *et al*., 1995). Accordingly our observation that gene *AQY1* is strongly induced indicates that aquaporin might be involved in the architecture of fluffy colonies. The growth strategy of low cell density allows the wild colony to occupy a wide territory quickly.

The ability to form fluffy colonies is not a fixed trait of wild *S. cerevisiae* and is regulated by environmental conditions. The switch from the fluffy to the smooth colonies in felicitous laboratory conditions seems to be connected to general reprogramming of cell life style (including changes in secretion, cell morphology, growth, etc.). Interestingly, no intermediate fluffy/smooth morphotype was observed, thus indicating the existence of a switching regulatory mechanism possibly resembling the interconversion of a/α mating type. Regarding microarray results,

one can hypothesize that changes in chromatin structure level are crucial for the switch. Previous observations indicating that *C. albicans* histone deacetylases Hda1p and Rpd3p as well as Sir2p protein (homologue of *S. cerevisiae* Sir2p) play roles in the phenotypic switching of *C. albicans* colonies (Perez-Martin *et al*., 1999; Srikantha *et al*., 2001) support this hypothesis. The fluffy/smooth differences in carbohydrate metabolism, transport, cell wall and aquaporin gene expression are evident in both 4-day-old and 7-day-old colonies, indicating that they might be directly connected to ECM production, cell–cell connection and colony surface properties. In contrast, differences connected with transposons and telomeric genes and, especially, differences in vitamin metabolism and cell cycle and polarity, are less significant or absent in 7-day-old colonies. Therefore, these genes seem to be more important in the earlier phases of fluffy colony development, when primary differences in colony morphology are just established.

In contrast to pseudohyphal transition, by which whole cell population responds to nitrogen starvation (Gimeno *et al*., 1992), when first grown on rich medium, most of the fluffy cells still preserved their original, fluffy life style. Only later, when laboratory growth conditions persisted, did the fluffy cells start to adapt one by one, i.e. to switch to the less energy-consuming smooth life style. This implicates the basic question of when, and in which cells, the switch occurs. It might be that some cells within the growing fluffy colony register the environmental modification, switch and, after replating, initiate smooth colony formation. Our preliminary observation of small smooth sectors within fluffy colonies supports this possibility. On the other hand, one cannot exclude the possibility that each individual plated cell is able to decide whether to form the fluffy colony or to switch to the smooth one. Further determination of the internal and external parameters that influence the efficiency of the fluffy-to-smooth transition and the search for 'natural' conditions enabling the reverse process might help to reveal the mechanisms involved.

The present work, together with our previous observations of differentiated gene expression within *S. cerevisiae* colonies (Minarikova *et al*., 2001) and intercolony ammonia signalling (Palkova *et al*., 1997), indicates that yeast cells do not only behave as individuals exhibiting a limited repertoire of responses against changing environmental conditions. On the contrary, yeasts are able to interact, to change their social behaviour according to environmental conditions (as demonstrated by the fluffy-to-smooth switch) and to create colonies that allow yeast cells to differentiate for the benefit of the whole population. It would not be surprising if such processes as apoptosis, still being questioned in yeast individuals (Gershon and Gershon, 2000), were finally to be found as the natural fate of particular cells within yeast colonies, reminiscent in several aspects of multicellular tissues.

Experimental procedures

Strains and media

Laboratory strain *S. cerevisiae* GRF18 (*MAT*α, *his3*Δ*1*, *leu2*Δ*0*) and brewery strain P23 are from the DMUP collection (Charles University, Faculty of Science, Prague, Czech Republic). Wild strain BRAIN 97 (BR) is from the Collection of the Chemical Institute of the Academy of Science, Bratislava, Slovak Republic (cat. no. CCY 21-4-97). Colonies were grown on GMA (3% glycerol, 1% yeast extract, 2% agar), MnMA (3% mannose, 1% yeast extract, 2% agar) or YEGA (1% yeast extract, 2% glucose, 2% agar). Liquid cultures were grown in GM (GMA without agar) or MnM (MnMA without agar). $CaCl_2$ (30 mM) was added to each medium used in the cell adherence assay.

ESEM analyses

Whole yeast colonies including about 2 mm of the surrounding agar were placed directly at the cryostage of the environmental scanning microscope AquaSEM (Tescan) and observed under 500 Pa at −18°C using an ionization detector at 15 kV. Two protocols were used to reach the final observation conditions. (i) In the structure-preserving protocol, the sample was chilled to 0°C in the chamber of the microscope under normal pressure. After 2 min, the vacuum was applied, and the temperature was simultaneously lowered to −18°C. Using this protocol, the three-dimensional structure of the colony was well preserved, resembling the *in vivo* structure revealed by optical microscopy and close-up photography. (ii) To improve the visualization of ECM, an alternative matrix-preserving protocol was developed. The sample was frozen to −10°C in the chamber of the microscope under normal pressure. After complete freezing of the agar (2–3 min), the vacuum was applied, and the temperature was simultaneously lowered to −18°C. Despite this protocol producing artificial fissures and magnified intercellular spaces in the colony, the ECM is clearly visible.

Pulsed-field gel electrophoresis

DNA samples were prepared from $\approx 2.5 \times 10^7$ cells as described previously (Janderova and Sanca, 1992). The Gene-line system TAFE (Beckman) was used, with the operating mode recommended for migration of *S. cerevisiae* chromosomes (stage I: 170 mA, switching time 4 s, 30 min; stage II: 150 mA, switching time 60 s, 18 h; stage III: 150 mA, switching time 30 s, 6 h).

Cell adherence assay (modified according to Stratford et al.*, 1988)*

Cells from liquid medium (2 day-old cultures) or cells from colonies (4 day-old colonies) suspended in water (both at concentrations of $1–4 \times 10^8$) were harvested by centrifuga-

tion, washed twice with 5 ml of water or 50 mM citrate buffer, pH 3.0, 5 mM EDTA (with vigorous mixing for 30 s) and suspended again in 2 ml of the same solutions. The presence of individual cells and cell aggregates was observed under the microscope.

RNA isolation and Northern blots

For total RNA isolation, either BR-fluffy or BR-smooth monocolonies growing on GMA were suspended directly in TES buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS). Approximately 800 monocolonies (4 days old) or 500 monocolonies (7 days old) were mixed for one RNA preparation. The exact procedure of RNA isolation is described at http://www.biologie.ens.fr/fr/genetiqu/puces/protocoles_puces.html. The RNA samples were quantified on a spectrophotometer. For Northern blot, 15 μg of total RNA was loaded. The radioactive probes for *FLO1*, *FLO10*, *FLO5*, *FLO9*, *HMS1*, *INO1*, *MAL32*, *SNQ2*, *SOR1*, *SPS100*, *TIP1*, *YMR045c*, *ACT1* and *YAR033w* were obtained by random priming on the complete open reading frame (ORF) [amplified by polymerase chain reaction (PCR) from the Research Genetics bank of yeast ORFs] using the Nonaprimer kit. For the *FLO11* probe, we used a PCR fragment corresponding to the last 1382 bp of the *FLO11* gene. The *ACT1* and *SNQ2* probes were used as invariant controls to normalize the radioactive signals for all probes.

Microarray analyses

Microarray slides containing most of the yeast ORFs (5885 PCR products) were obtained from Hitachi Software and DNAChip Research. Two micrograms of mRNA was used for each reverse transcription reaction. Detailed protocols are described at http://www.biologie.ens.fr/fr/genetiqu/puces/protocolespuces.html. The arrays were read by a Genepix 4000 scanner (Axon) and were analysed with the GENEPIX 3.0 software. Each microarray result presented here is an average of at least 10 independent biological measurements.

Biocomputational analyses of microarray data

We excluded artifactual spots, saturated spots and low signal spots. Assuming that most of the genes have unchanged expression, the Cy3/Cy5 ratios were normalized using the median of all the ratios for each experiment.

ECM extraction and analysis

Colonies (7 days old) were suspended in PBS (8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4 and 0.24 g l^{-1} KH_2PO_4, pH adjusted to 7.4), vortexed briefly, and the suspension was incubated for 10 min on a roller and centrifuged at 5000 *g*. For sequential extraction, sediment was again extracted in a sequence of PBS–1% Tween and PBS–1% SDS. PBS extracts were treated with either 10 units of PGNase F (NEB; no. P0704S) overnight (reaction conditions according to NEB Technical Bulletin, http://www.neb.com/) or proteinase K (1 mg ml^{-1} or 0.1 mg ml^{-1} concentration) for 1 h at 37°C. Proteins were precipitated from extracts according to the methods described by Wessel and Flugge (1984) and analysed on 10% SDS-PAGE (Sambrook *et al*., 1989). For glycoprotein analysis, PAGE gel was blotted on polyvinylidene difluoride (PVDF) membrane, and glycoproteins were visualized by sequential incubation in concanavalin A, horseradish peroxidase and chloronaphthol–H_2O_2 solution as described previously (Hawkes, 1982).

Photography

Colonies were photographed with illuminating light coming through the plate from the bottom (Fig. 1, day 1) or illuminated from above (Fig. 1, days 3 and 6). A Hitachi HV-C20 colour camera with a Navitar objective, Fiber-Lite PL-800 illumination system and Lucia G/F software (Laboratory Imaging) were used. One-day-old colonies and cells (Fig. 1) were photographed using an Olympus BX60 microscope.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3332/mmi3332sm.htm.

Table S1. Detailed results of microarray experiments.

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