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Article *in* Annals of Microbiology · January 2002

4 authors, including:

Andrea Pulvirenti
Università degli Studi di Modena e Reggio Emilia

M. Antonio Todaro
Università degli Studi di Modena e Reggio Emilia

Paolo Giudici
Università degli Studi di Modena e Reggio Emilia

Ann. Microbiol., **52**, 245-255 (2002)

Interspecific hybridisation by digestive tract of invertebrates as a source of environmental biodiversity within the *Saccharomyces cerevisiae*

A. PULVIRENTI[1]*, C. ZAMBONELLI[2], A. TODARO[3], P. GIUDICI[1]

[1]Dipartimento di Scienze Agrarie, Università degli Studi di Modena e Reggio Emilia, Via Kennedy 17, 42100 Reggio Emilia; [2]Dipartimento di Protezione e Valorizzazione Agroalimentare (DIPROVAL), Università degli Studi di Bologna, Villa Levi, Via Fratelli Rosselli 107, 42100 Reggio Emilia; [3]Dipartimento di Biologia Animale, Università degli Studi di Modena e Reggio Emilia, Via Campi 213/d, 41100 Modena, Italy

Abstract - In order to verify whether animals can promote the formation of new yeast strains by increasing the chance of encounters between otherwise segregate spores within persistent asci, three invertebrate species representative of the marine, freshwater and terrestrial ecosystems were fed with two different diets composed of one or a combination of two strains of sporulated yeast and the egested material was analyzed for evidence. The digestive apparatus of the tested species is able to break open the asci wall (most probably by enzymatic action) produced in the species belonging to the *Saccharomyces sensu stricto* group without affecting the viability of the spores contained therein. The spores ejected with the fecal material have a high capacity for agglutination and, in the presence of favorable conditions, can germinate directly or conjugate to form hybrids. Hybrids between strains of the same species give rise to cultures in which the parent characteristics have new combinations. The interspecific hybrids *Saccharomyces cerevisiae* × *Saccharomyces uvarum*, although sterile, can propagate by asexual reproduction. These results support the hypothesis of horizontal transfer of genetic material between yeast species and the likelihood of hybrid formation in natural settings, and possibly shed light on the high biodiversity of yeast observed in nature.

Key words: yeasts; hybrids; ITS; ecology; horizontal transfer.

INTRODUCTION

Saccharomyces cerevisiae and other yeasts belonging to the *Saccharomyces sensu stricto* group were subjected to a number of genetic studies, using the classic technique between spores in the case of the homothallic strains and conjuga-

*Corresponding author. Phone +39-0522383232; Fax +39-0522301482; E-mail: pulvirenti.andrea@unimo.it

tion between haploid cells in the case of the heterothallic strains (Winge and Lausten, 1939; Winge and Roberts, 1958). These techniques were also used with the aim of genetic improvement to obtain new strains of interest to winemaking (Romano *et al*., 1985; Thornton, 1985) and to develop interspecific hybrids (Zambonelli *et al*., 1993; Zambonelli *et al*., 1997; Kishimoto, 1994; Marinoni *et al*., 1999). To these ends, the method involving conjugation between spores has proved to be more suitable because, as demonstrated by Thornton and Eschenbruch (1976), the natural *Saccharomyces* wine yeast cells are homothallic. Intra- and interspecific hybridization also occurs in nature, and interspecific *Saccharomyces* hybrids were found by Masneuf *et al*. (1998) in wine and cider. The cross and resulting hybrids (not necessarily fertile) between strains belonging to species of the *sensu stricto* group are possible because the organization and order of chromosomes seem to be fairly preserved. Therefore, the chromosomes are believed to be at least partially homologous (Hunter *et al*., 1996; Ryu *et al*., 1996). In interspecific hybrids of the *Saccharomyces sensu stricto* group, the number of bands of electrophoretic profiles of entire chromosomes is greater than that of the parent strains. In fact, the hybrids contain both parental sets of chromosomes but the mtDNA from only one parent (Giudici *et al*., 1998; Marinoni *et al*., 1999; Pulvirenti *et al*., 2000a).

The liberation of intact spores is necessary so that hybrids can be formed between different strains or species of homothallic yeast cells. However, yeasts belonging to the *Saccharomyces* genus form asci with stable walls that do not free the spores, a key trait in the taxonomy of the group (van der Walt, 1970).

Yeast is a natural food item for many invertebrate animals and several species are routinely reared in the laboratory on yeast-based diets, usually baker's yeast *Saccharomyces cerevisiae* (Fukusho *et al*., 1980; Lotufo, 1997; Lubzens *et al*., 2001). It is assumed that, in order to derive the most nutrients from this food source, the animal's digestive apparatus should be able to break open the walls of the cells, thus releasing cytoplasmic material. Likewise, if ingested, the cell and ascus walls can be broken by the enzymatic hydrolysis of the polysaccharides that comprise them. The enzymes responsible for the hydrolysis are known industrially as Zymolase, Lyticase and Helicase, the latter being obtained in large quantities from the gastropod *Helix pomatia*. On these grounds, it is possible to it can be assume that, in nature, the spores from the asci of *Saccharomyces* can be released in the digestive tracts of these animals and that some of them are released intact into the environment enhancing the chances of hybrid formation.

This study was carried out to determine: i) if animals that feed on yeast can hydrolyze the ascus walls of the *Saccharomyces* taxon without affecting the viability of the spores; and ii) if the spores released into the environment can give rise to natural hybrids. The final aim was to find out whether the horizontal transfer of genetic material between different species is possible. In order to verify these hypotheses, three invertebrate species, representative of the marine, freshwater and terrestrial ecosystems, were fed with two different diets composed of one or a combination of two sporulated yeast strains and the egested material was analyzed for evidence of genetic exchange.

MATERIALS AND METHODS

Yeast strains. The yeast strains used all belong to the DIPROVAL collection of the University of Bologna (Italy), and all of them have specific characteristics: a) strain *S. cerevisiae* No. 7070 is a non-producer of hydrogen sulfide and forms a white streak in the culture medium containing bismuth sulfide as indicator; moreover, this strain grows at temperatures above 37 °C and does not ferment melibiose; it should be pointed out that, for this strain as well as for the others, a single spore culture was used (7070-1A) with homozygote characteristics of the parental strain; b) strain *S. cerevisiae* No. 6167 (single spore culture 6167-1A) produces hydrogen sulfide and forms a black streak; this strain also grows at temperatures above 37 °C and does not ferment melibiose; c) strain *Saccharomyces uvarum* No. 7877 (single spore culture 7877-1A) is a non-producer of hydrogen sulfide; this strain does not grow at 37 °C and ferments melibiose; d) intraspecific hybrid No. 839, obtained from a cross between strains *S. cerevisiae* 7070-1A and 6167-1A, is a heterozygote as regards the capacity to produce hydrogen sulfide; the hybrid forms hydrogen sulfide and segregates the character with a constant 2:2 ratio, on the basis of then tetrads; e) interspecific hybrid *S. cerevisiae* x *S. uvarum*, obtained by crossing strains 7070-1A and 7877-1A; the hybrid forms a brown streak in a bismuth sulfide culture medium because it produces a low quantity of hydrogen sulfide; it grows at temperatures above 37 °C and ferments melibiose. This hybrid sporulated with high efficiency, but it is sterile and its spores do not germinate. The strains used and their hybrids are described in earlier studies (Zambonelli *et al*., 1993; Zambonelli *et al*., 1997; Giudici *et al*., 1998; Pulvirenti *et al*., 2000b). A preliminary check on the sterility of the spores produced by the interspecific hybrid 7070-1A x 7877-1A was carried out on isolated spores obtained directly by breaking the asci wall with a de Fonbrune's micromanipulator. A similar analysis of tetrads of the intraspecific hybrid 839 was performed to test the goodness of the chosen marker (hydrogen sulfide production).

Nutritive media. Basic medium for fecal material suspension: 10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 20 g l^{-1} glucose (YPD, liquid or solid - 20 g l^{-1} agar).

Selective medium for interspecific hybrid enrichment: YPD with melibiose (30 g l^{-1}) instead of glucose (YPMel).

Sporification medium: Mc Clary's Acetate Agar composed of: 1 g l^{-1} glucose, 1.8 g l^{-1} KCl, 2.5 g l^{-1} yeast extract, 8.2 g l^{-1} sodium acetate, 15 g l^{-1} purified agar.

BiGGY agar (Oxoid) containing bismuth sulfide for the determination of the "capacity to produce hydrogen sulfide" marker character. In this medium, the H_2S-producing strains form a light brown and dark brown streak, whereas the non-H_2S-producing strains form a white streak.

Diets fed to the organisms. The diets were as follows: diet A, containing only the hybrid 839; diet B, mixture of strains 7877-1A and 7070-1A.

Experiment 1. Species: *Tigriopus fulvus* (Crustacea, Copepoda, Harcacticoida). This is a 1 mm long marine species, inhabiting the splash pool habitat of the entire Mediterranean basin. The genus is cosmopolitan in distribution. In natural settings, *T. fulvus* feeds on micro-algae and detritus and produces fecal material in

the form of fecal pellets of about 50 × 30 μm in size. Laboratory populations can be maintained using vessels of different capacities and yeast suspensions as food (Carli *et al*., 1995; Todaro *et al*., 2001).

The experimental setup included six 50 ml vessels arranged in two series, one for each diet. Five adult copepods per vessel were allowed to feed *ad libitum* for 4 hours, then rinsed with purified sea water and transferred to new, yeast-free incubation chambers; the fecal pellets produced during the first two hours were collected using a micropipette and discarded, whereas the subsequent pellets were transferred to an appropriate culture medium.

Experiment 2. Species: *Tubifex tubifex* (Annelida, Oligochaeta, Tubificidae). This 2-7 mm long freshwater worm is a cosmopolitan species that forms particularly abundant populations in organically enriched sediment. Like other tubificids, it feeds continuously in a conveyor-belt fashion by ingesting particles in bulk at depth and defecating on the sediment surface. Feces appear in the form of small tubings. Laboratory cultures are maintained using fine sediment (< 125 μm grain size) and a variety of food including yeasts (Marian and Pandian, 1984).

Six 50 ml polypropylene centrifuge tubes (11.5 x 2.7 cm) arranged in two series were used for the experiment. The tubes of each series were three-quarter filled with previously autoclaved fine sediment mixed with 0.5 g of appropriate yeast diet, and one-quarter topped with sterile spring water. Fifteen worms with the digestive tract previously cleansed were placed on the sediment surface of each tube and allowed to burrow into the sediment for 8 hours. At the end of this period, the water above was removed. The sediment surface was then covered with a thin layer of polyester aquarium floss and a circle of damp cheesecloth. The floss and the cheesecloth were held in place by a PVC split ring. Each tube was then filled with sterile spring water. After a few hours, the *T. tubifex* began to extrude their posterior ends through the floss and cloth to eliminate fecal tubing. Feces produced during the initial 12 hours were discarded. Thereafter the feces were transferred into the appropriate culture medium.

Experiment 3. Species: *Drosophila melanogaster* (Insecta, Diptera) larvae, the well-known cosmopolitan fruit fly. In their natural environment, mature females are attracted by the smell of fermenting vegetables, generally fruit, in which they then lay eggs. The larvae feed on the ripe pulp and produce feces in the form of loose tubing. Mass culture of this species can be maintained in the laboratory under different diet regimes (Krebs *et al*., 2001).

Six Petri dishes (2.2 cm in diameter) were used for this experiment and arranged into two series according to the diet (A and B). The dishes contained a 3 mm thick layer of 4% sterile agarose gel blanketed with the appropriate food. Five larvae that had been rinsed with sterile water were placed on each dish. To avoid possible contamination, larvae were used that represented the third generation of flies cultured in tow series of glass jars (six in total) containg autoclaved fruit pulp combined with diet A and B as food. The food was allocated according to the final destination of the larvae (e.g. larvae from culture using diet A were transferred to dishes of the A series etc.). Larvae were allowed to feed overnight, then rinsed with sterile spring water and transferred to clean dishes; feces produced during the first two hours were discarded, while those produced thereafter

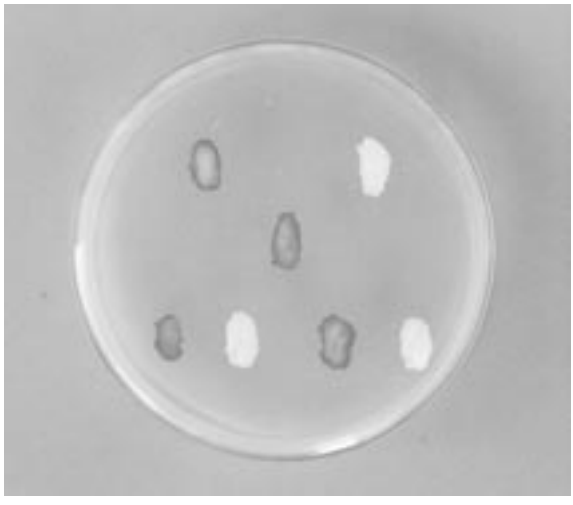

FIG. 1 – Testing of the marker character validity in BiGGY agar medium. Black and white streaks are formed respectively by producing and non-producing H_2S strains respectively. a) parental strain 6167-1A; b) parental strain 7070-1A; c) hybrids 839; d) single spore cultures obtained from one tetrad of the hybrid 839.

feces were collected using micropipettes and transferred to the appropriate culture medium.

Isolation of yeasts. The fecal pellets obtained from the animals fed on hybrid 839 (*S. cerevisiae x S. cerevisiae*) were collected under sterile conditions and suspended in liquid YPD, homogenized, suitably diluted and inoculated in a Petri dish to check the hydrogen sulfide production. Tetrad analyses confirmed that the marker character was constantly segregated by a ratio of 2:2 (Fig. 1).

The fecal pellets collected from the specimens fed on the two parental strains, 7070-1A (*S. cerevisiae*) and 7877-1A (*S. uvarum*), were collected, homogenized, suspended in liquid YPD and incubated at 25 °C. After 24 hours, 1 ml of sample was transferred to a beaker containing liquid YPMel and incubated at 40 °C. The presence of only melibiose and a temperature of 40 °C are restrictive conditions that allow only the growth of hybrids between *S. cerevisiae* and *S. uvarum*. In fact, the capacity to assimilate melibiose is a characteristic trait of *S. uvarum*, while the species cannot grow at temperatures above 37 °C. The strains belonging to species *S. cerevisiae*, on the other hand, are incapable of assimilating melibiose and grow at 40 °C. The growth of strains in liquid YPMel incubated at 40 °C is indicative of the presence of certain interspecific hybrids (Zambonelli *et al*., 1993; Zambonelli *et al*., 1997; Giudici *et al*., 1999).

Identification and checking of the strains isolated. The strains isolated were identified using the PCR/RFLP analysis of the ITS regions, which is capable of differentiating of the species within the genus *Saccharomyces sensu stricto*, using

primers ITS1 (5' – TCC GTA GGT GAA CCT GCG G – 3') and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC – 3'), by means of the procedure described earlier (Esteve-Zarzoso *et al*., 1999; Pulvirenti *et al*., 2001). The intraspecific hybrids were identified by Pulsed Field Gel Electrophoresis. Conditions for the preparation of the plugs were as those described earlier for *S. cerevisiae* (Vezinhet *et al*., 1990), with a few modifications (Nguyen and Gaillardin, 1997). To visualize the whole karyotype, chromosomes were separated on a CHEF MAPPER apparatus (BioRad, Richmond, California, USA).

RESULTS

Analysis of the interspecific hybrids 7070-1A x 7877-1A confirmed their sterility, as they produced numerous asci, even with four spores, but these did not germinate. Both parents are H_2S negative; however, by complementation, their hybrids are H_2S positive because they produced brown instead of white streaks in the BiGGY culture medium. Microscopic analysis of the fecal material egested by the three species revealed that, in all cases, the walls of the most asci had been broken down and the spores set free. The free spores are clearly visible, especially those still joined in groups of 3 or 4 with the same arrangement as in the asci (Fig. 2). A few intact cells which were formed mainly by yeast residuals, were visibly interspersed within the fecal material. Ten free spores from hybrid 839 (*S. cerevisiae* x *S. cerevisiae*) were directly isolated using the micromanipulator and were found to be viable; six of these formed a white streak in BiGGY and four formed black streaks. Whether the rupture of the asci and vegetative cells was caused by the mechanical action of enzymatic hydrolysis or by a combination of the two is still open to discussion. However, an examination of the material confined within the different tracts of the intestine of the *Drosophila* larvae indicates that, in this species at least, the asci breakdown begins in the mid-intestinal region, therefore pointing to a predominant enzymatic cause.

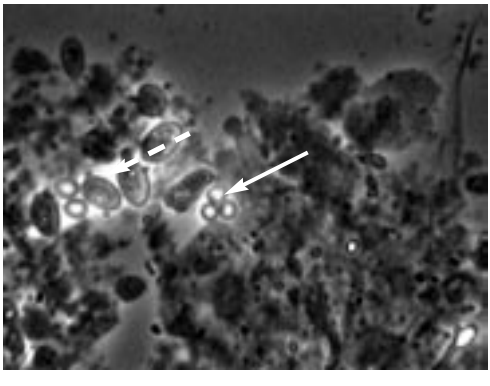

FIG. 2 – Microscopic observation of fecal pellets *of Drosophila melanogaster*. In the fecal pellet formed mainly by yeast debris, free spores (————>) and intact cells (– – – – – –>) are recognizable.

Yeast cells isolated from the feces of *Tigriopus fulvus* fed with diet A (hybrids 839) showed the segregation of the trait "H_2S production" in the ratio 15/85 (white/black). The predominant presence of black strains in BiGGY compared to the expected 2:2 ratio may be due to a combination of two reasons: i) the carry-over of hybrid cells along with the fecal material; or ii) the persistence of undigested hybrid cells in the fecal material. The white cultures in BiGGY certainly derive from the hybrid spores; however, it is possible that they are formed from spores still contained inside asci with intact walls and not from free spores. For this reason, the result, although positive, is not sufficient to affirm that the spores of *S. cerevisiae* are released in the digestive tract. Incubation of fecal material from the copepod fed with diet B did not show any evidence of hybrid formation.

In accordance with the *Tigriopus fulvus* experiment, the feces obtained from the freshwater worms fed with diet A and directly spread onto YPD agar showed the segregation of the H_2S trait; in this case, the white/black ratio was 10/90. However, there was no positive result concerning hybrid formation when fecal material obtained from *T. tubifex* fed with diet B was incubated.

When spread directly onto YPD agar, feces produced by *D. melanogaster* larvae fed with the hybrid 839 (diet A) when directly spread onto YPD agar, resulted in both white and black colonies (ratio 15/85), once again indicating the occurrence of segregation of the two parental strains.

In this experiment, a positive result was also obtained by incubating the material egested by the larvae grazing on diet B. In fact, it was possible to isolate the interspecific hybrid *S. cerevisiae* x *S. uvarum*. To obtain the hybrid, however some further isolation steps were necessary. The process was as follows: the fecal material egested required initial enrichment in liquid YPD to allow germination of the free spores and their subsequent conjugation. It then became necessary to carry out a selection in YPMel following incubation at 40 °C to inhibit the growth of cells whose lineage derived from single parental cells. In fact, only the hybrids that formed can grow under these conditions, and not *S. cerevisiae* or *S. uvarum*. Aliquots of the samples were inoculated on plates containing YPMel and incubated at 40 °C. There were a large number of brown cultures in the BiGGY agar, and these putatively are hybrids. The results were confirmed by PCR/RFLP of the ITS region using restriction enzyme *Hae III*, which has a high capacity of discrimination between strains of the *S. cerevisiae* and *S. uvarum* species, giving rise to a banding pattern of four fragments corresponding to 320, 220, 180 and 145 bp in the former species and a profile of three fragments (500, 220 and 145 bp) in the latter. The profile, shown in figure 3 and pertaining to the putative hybrid is as such and derives from a conjugation between parental strains *S. cerevisiae* and *S. uvarum*. In fact, the restriction profile (number and size of bands) is the result of a combination of the two parents. These results are also confirmed by PFGE (Fig. 4). The karyotype of the putative hybrid shows a typical additional pattern which is in agreement with the literature on hybrids of *S. cerevisiae* x *S. uvarum* (Giudici *et al*., 1998; Pulvirenti *et al*., 2000a).

The high percentage of brown cultures in BiGGY agar are all putatively interspecific hybrids, and this is not due to their actual dominance, but only because of the enrichment medium, which distinctly favors them above the parental strains. However, merely the fact that they are present is sufficient to support the hypothesis that interpecific hybrids can also be formed in nature. In fact, in this experi-

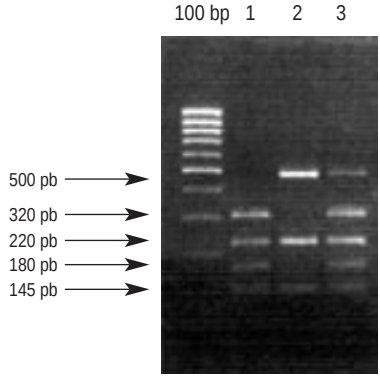

FIG. 3 – *Hae*III restriction patterns of ITS regions of the hybrid and its parental strains. Lane 1: *S. cerevisiae* 7070-1A; lane 2: *S. uvarum* 7877; lane 3: hybrid.

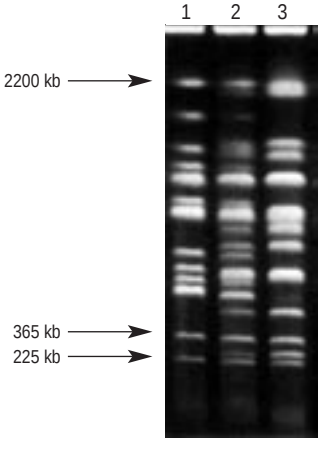

FIG. 4 – Contour-Clamped Homogeneous Electric Field (CHEF) banding patterns of chromosomal DNA of hybrid and its parental strains. Lane 1: *S. uvarum* 7877-A.; lane 2: hybrid; lane 3: *S. cerevisiae* 7070-1A.

ment, the hybrid was derived from the conjugation between spores freed in the digestive tract of the animals.

DISCUSSION

One of the possible ways, and probably the most important, for yeasts of the *Saccharomyces* genus to give rise to natural intra- and interspecific hybrids is through the digestive tract of various animals that are capable of hydrolyzing the

asci walls and releasing free spores into the environment. The spores have a high capacity for agglutination and, in the presence of favorable conditions, they can germinate directly or conjugate to form hybrids.

Hybrids between strains of the same species give rise to cultures in which the progeny have characteristics that differ from those of the parents, which explains the biodiversity often seen in studies of yeast in grapes e.g. Castelli (1954) and Masneuf *et al*. (1998). It is worthwhile to keep in mind that, in principle, the definition of a yeast species should be based on the concept of genetic isolation, as in the case of other eukaryotic organisms. However, yeast cells belonging to the genus *Saccharomyces sensu stricto* can conjugate and create hybrids in both in experimental conditions and in nature. These hybrids can propagate by asexual reproduction.

Of the animals that feed on yeasts, certain insects, such as midges, bees, wasps and fruit flies, are of special importance. These animals most likely are responsible not only for natural hybridization, but also for yeast dispersal into the environment and for the exchange of yeast strains between grape bunches from different vineyards (Mortimer and Polsinelli, 1999). In addition, there is also the fact that grapes constitute the main food for many birds and it may be possible that an exchange of yeast cell cultures could also occur in these manner.

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