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General evidence supporting the hypothesis that *Saccharomyces cerevisiae* vaginal isolates originate from food industrial environments

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SUMMARY

Saccharomyces cerevisiae strains isolated from pregnant women were identified and characterized by molecular techniques which disclosed a wide chromosomal variability and possible segregations due to sporulation. The morphological analysis showed that very few strains were able to sporulate and generate pseudohyphae, whereas none produced proteases, raising some doubts on the importance of these characters in strain pathogenicity. The analysis of ethanol production revealed that these strains are quite similar to those found in fermentative plants, suggesting a possible derivation from the food industrial environment.
Since the absence of relevant amounts of sugar does not confer selective advantage to strong fermentative metabolisms, these findings suggest that a metabolic adaptation to the vaginal environment did not occur yet.

KEY WORDS: yeast, *Saccharomyces,* vaginal environment, physiology, morphology, adaptation

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INTRODUCTION

S. cerevisiae is traditionally used in wine, bread and beer production and now in biotechnological applications too. Although it is essentially a non-pathogenic yeast, rare cases of vaginitis, endocarditis and fungemia have been associated with this microorganism especially, although not exclusively, in severely immunocompromised hosts.

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Yeast vaginites are normally caused by *C. parapsilosis*, *C. tropicalis, C. glabrata* and mainly by *Candida albicans.* The incidence of *S. cerevisiae* vaginitis has been estimated to be lower than 1%. A different situation has been reported in Italy with new cases ranging between 3% and 5% of patients (Agatensi*, et al.*, 1991). Vaginitis caused by *S. cerevisiae* is defined as the presence of vulvovaginal symptoms and signs of inflammation in patients from whose vagina *S. cerevisiae* is isolated in pure culture and in whom all other causes of vulvovaginitis have been excluded.
Recent investigations have assessed the pathogenicity of clinical and non-clinical isolates in an attempt to elucidate the mechanisms by which this microrganism causes disease. Certain phenotypic traits have been associated with increased virulence, such as the ability to grow at 42°C and increased pseudohyphae production.

Aim of our study was to characterize some *S. cerevisiae* strains isolated from asymptomatic patients and to compare them with those normally present in food industrial environments to ascertain whether these yeasts are similar to those from the outer environment or are undergoing some type of microevolution triggered by selection for the specific vaginal environment. We focused our attention on pseudohyphal morphology and on the ability to produce ethanol, a trait quantitatively present in the wine and beer industry, but less pronounced in other situations.

MATERIALS AND METHODS

Strains

The cultures were collected in the Perugia hospital from 610 women with clinically diagnosed vaginites, during a four month period. Immediately after isolation, on the basis of an API 20C analysis, with more than 80% confidence (BioMerieux, Marcy l'Etoile, France), a total of 12 putative isolates of *Saccharomyces cerevisiae* strains were obtained (1.96% of the vaginal specimens examined). After a further isolation the strains were maintained in 17% glycerol at -80°C). In order to confirm their pre-identification, an electrokaryotyping analysis was carried out, with the rationale that strains of the *sensu stricto* group of the genus *Saccharomyces* are easily recognizable by the presence of more than 12 chromosomal bands ranging from 2.200 to 230 Kb (Cardinali and Martini, 1994).

Growth conditions

Yeast cells were grown in YEPD (Yeast Extract 1%, Peptone 1%, Dextrose 2%) at 25°C unless indicated. Growth at 37, 39, 42°C was determined by streaking freshly grown cells onto plates and examining colony size (if any) after 1 or 2 days. Growth at temperatures >37°C was examined because such temperatures have been observed in febrile patients.

Yeast genotyping

Chromosomal grade DNA was extracted as previously described (Cardinali, *et al.*, 1995) and run in a CHEF Mapper apparatus (Bio-Rad - USA) under the following programme setup: time 24 h, angle 106°C, switch time 100 sec, voltage gradient 6,0 V/cm, intensity 110-130 mA, temperature 17.5°C. CHEF bands were analyzed as follows. Migration distances of the CHEF bands were calculated with NIH - Image 1.62 (http://rsb.info.nih.gov/nih-image/Default.html) and transformed into the corresponding molecular weight values by entering them into a polynomial regression equation obtained from the DNA standards with the Kaleida Graph programme. Molecular weights data were introduced into the ClassMaker 2.27 application (Cardinali, *et al.*, 2003) to obtain a binary classification (1= presence of the band; 0 = absence), using the CL2 algorithm with a threshold value T= 95. *Sma*I digestion of chromosomal DNA was obtained following a previously described procedure (Gordillo, *et al.*, 1993).

Morphological and physiological tests

The production of pseudohyphae was tested by microscopic inspection every second day according to the Dalmau Plate and to the agar covered slide techniques on Corn Meal Agar medium.
The sporulation was tested in acetate medium (Yarrow, 1998).
The determination of protease production was performed according to Aoki *et al.* (Aoki, *et al.*, 1990) on agar plates containing 33 mg/ml bovine serum albumin fraction V (Sigma Chem Co., St. Louis, Mo., USA). The plates were incubated at 37°C for 7 days.
Ethanol production was measured with ebulliometric system according to the routine procedure suggested by the manufacturer.

Statistical analyses

Physiological data of the environmental or food industrial *S. cerevisiae* strains were retrieved from the *"Industrial Yeasts Collection DBVPG"* at the University of Perugia (www.agr.unipg.it/dbvpg/home.html). Statistical analyses were carried out with the MS Excel application.

RESULTS

Yeasts genotyping

The strain MPG313 showed only six bands and was later classified as a *Candida albicans*, whereas the other twelve isolates presented the expected karyotype of *S. cerevisiae* (Fig. 1a). Eight dif-

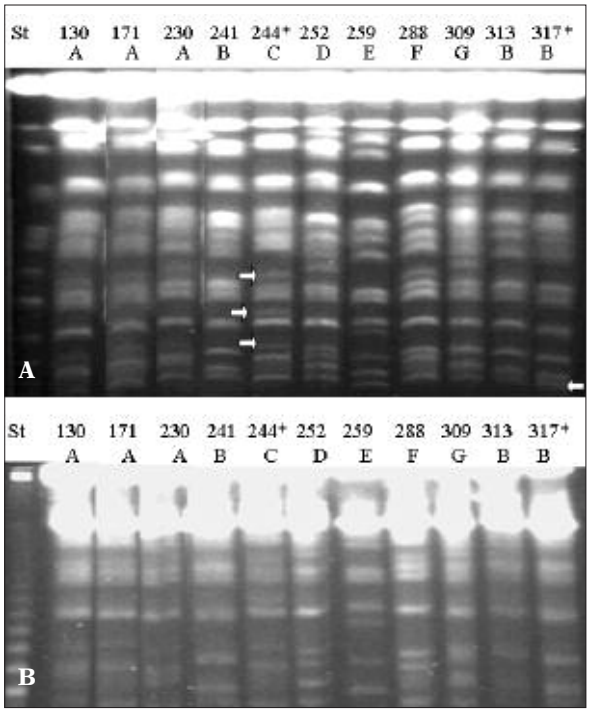

FIGURE 1 - *Genotypic analyses, CHEF (upper panel) and* Sma*I analysis (lower panel) of the vagina isolates. Arrows indicate changes in the banding pattern of two strains isolated from the same patient at two different times.*

ferent types, labeled with capital letters from A to H, could be discriminated. Only types A and B included more than one strain, namely the isolates MPG130, MPG171 and MPG 230 belong to type A, whereas type B incorporated the strains MPG241, MPG313 and MPG317. Types F and G were rather similar, differing by only two monomorphic bands of which one was present in the former and one in the latter, as indicated by the two triangles of figure 1. Interestingly, the two strains MPG244 and MPG317, isolated at two successive times from the same patient, differed by at least four chromosomal bands as indicated by the arrows in figure 1. This suggests that either the same strain underwent a chromosomal segregation between the two samplings or that two successive and independent infections occurred (Blasi*, et al.*, 2001). The karyotypic data were compared with the analysis of the *Sma*I digestion of the chromosomal DNA (Fig. 1b), finding that the digestion pattern matched with the electrokaryotypes. In fact the three strains of type A and the three of type B appeared identical, whereas all others differed by more than one band. The strong difference of strain 259 from the others was documented in both analyses.

Morphological and physiological tests

Only isolates 309 and 313 presented poorly branched pseudohyphae, like those shown in figure 2b, whereas all other strains displayed elliptical single cells (Fig. 2a). After two weeks, only

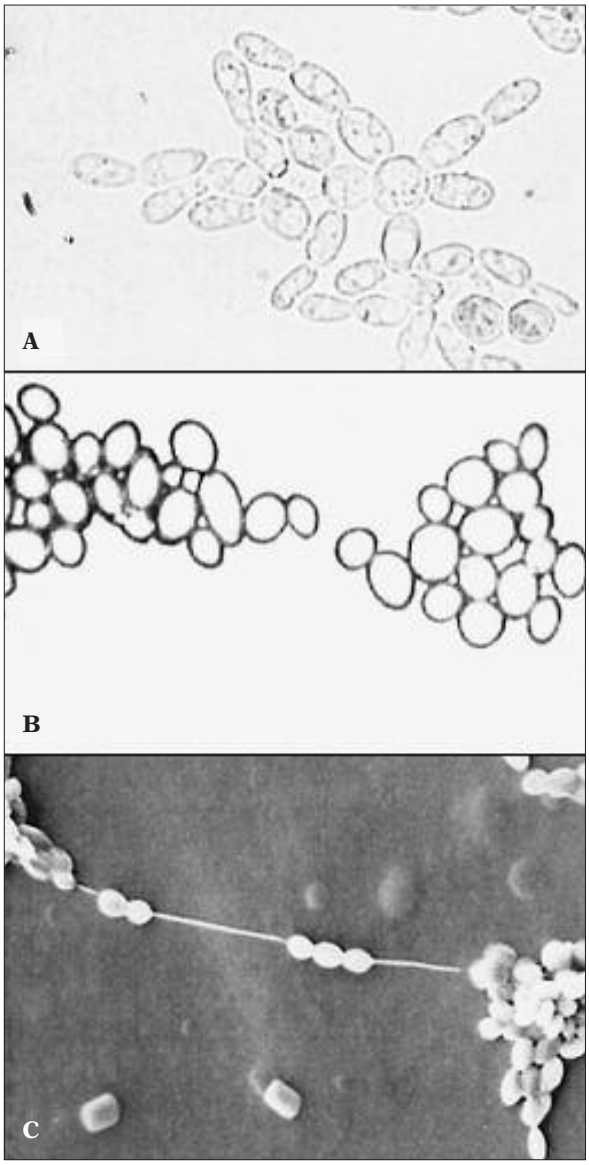

FIGURE 2 - *Micromorphology of the vaginal yeast isolates. a) pseudohyphal growth, b) growth as single cells, c) SEM image of a singular morphology found in the isolate MPG 213 (more details in the text).*

TABLE 1 - *Morphological analysis*

Strains	*Spore*	*Pseudohyphae*	*Growth at 42°C*
MPG130	-	-	-
MPG171	+	-	-
MPG230	-	-	-
MPG241	-	-	-
MPG244	-	-	-
MPG252	-	-	-
MPG259	+	-	-
MPG288	-	-	-
MPG309	+	+	-
MPG313	+	+	-
MPG317	-	-	-

four strains (MPG171, MPG259, MPG309, MPG313) exhibited ascospores.
No strain showed protease production.
Ethanol was yielded at concentrations ranging from 9.83%vol./vol. up to 13.60%vol./vol (Tab. 1) with an average of 11.87% vol./vol. and a standard deviation of 1.22. More than half the final alcohol was produced in the first five days, indicating a strong fermentative vigour typical of those cultures which are positively selected in the food industrial environment thanks to the rapid rate of ethanol production.

DISCUSSION

Together, the molecular data confirm the pre identification carried out with the API system and indicate some variability, possibly due to chromosomal segregation of heterozygote for the length of some homologs. However, the possibility that the chromosomal rearrangements should be ascribed to segregation requiring further studies, considering the low attitude to the sporulation showed by the isolates under study. The low sporulation (33%) was unexpected. *S. cerevisiae* strains are known to sporulate well on isolation, although they tend to lose this ability after some months in collection.
The fact that most of the cultures did not form pseudomycelium is not surprising, because this feature is variably present in the *sensu stricto* group of the genus *Saccharomyces* (Vaughan-Martini & Martini, 1998). However, these findings suggest that the filamentous growth and the ability to produce proteases cannot be considered important pathogenic factors as in *C. albicans* (De Bernardis, *et al.*, 1993; Mitchell, 1998; Phan, *et al.*, 2000; Umeyama, *et al.*, 2005).
Although it is not a *S. cerevisiae*, and does not attain to this study, we have examined the pseudohyphal growth of the strain MPG313,

TABLE 2 - *Ethanol production (%vol/vol)*

Days	*MPG130*	*MPG171*	*MPG230*	*MPG241*	*MPG244*	*MPG252*	*MPG259*	*MPG288*	*MPG309*	*MPG313*	*MPG317*
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	1.07	1.34	1.05	1.25	0.78	1.16	1.12	1.17	0.62	1.09	0.69
2	3.93	4.29	3.88	3.92	3.79	4.33	3.74	3.77	3.47	3.90	3.70
5	8.22	8.42	7.44	8.01	8.37	9.81	8.42	7.78	7.53	7.64	8.56
6	9.24	9.31	9.08	8.94	9.27	10.91	9.45	8.70	8.19	8.21	9.70
7	10.04	10.07	10.00	9.79	10.00	11.82	10.38	9.59	8.78	8.74	10.77
9	11.13	11.09	11.25	10.88	10.88	12.80	11.77	10.73	9.43	9.45	13.10
12	11.91	11.77	12.28	11.68	11.50	13.60	13.14	11.52	9.83	9.99	13.35

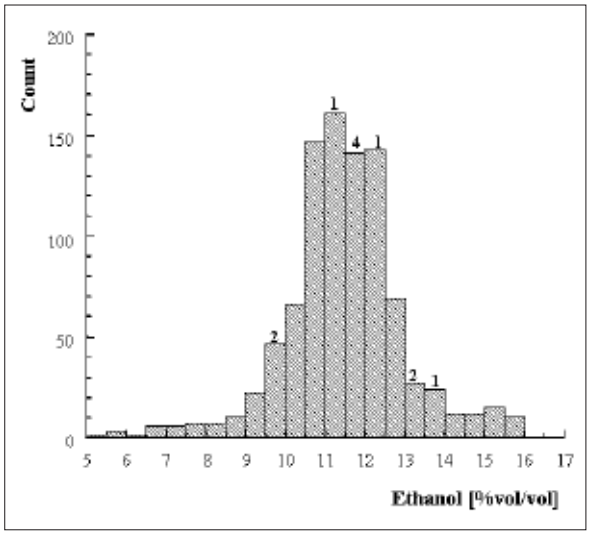

FIGURE 3 - *Distribution of the food industrial and environmental S. cerevisiae strains of the DBVPG collection. Numbers on top of the histogram indicate how many vaginal isolates fall into the single classes.*

finding a strange morphology, shown in figure 2c, where a long cord connects two large clusters of budding cells. Interestingly, two small groups of aligned cells are interpolated in the cord. Further analyses, carried out by confocal microscopy, suggested that the internal part of the cord is hollow (data not shown). To our knowledge, this is the first news of such a morphological structure, whose specific function is currently unknown.

Ethanol production data of 936 strains from the DBVPG collection show an almost normal distribution (Fig. 3) ranging from 5% v/v to 16% v/v, with an average of 11.27% v/v. and a standard deviation of 1.61. The matching of these descriptive figures with those of the vaginal isolates is further confirmed by the fact that all the vaginal cultures diverge from the mean by the standard deviation (9.66 to 12.88) with the exception of three strains yielding more than 13% v/v ethanol.

The species *S. cerevisiae* includes strains with the ability to produce more than 15% v/v ethanol, although some strains cannot yield more than 7%. Results from different studies suggest that strains characterized by high fermenting power are more often isolated from food industrial places, like wineries, probably due to the high selective pressure typical of industrial fermentation (Martini, 1993; Martini and Ciani 1996; Corte, *et al*., 2006).

The rationale of studying the fermentative power of vaginal *S. cerevisiae* cultures is that their adaptation to an environment poor in fermentable sugars like the vagina should decrease the selective pressure and favour the emergence of strains with little fermentative power, similar to those already found in environments different from the sugar fermentation industries.

Together, these data seem to indicate that the strains examined are physiologically similar to the best performing strains of the species, suggesting that no special adaptation to the vaginal environment took place and that these cultures could be derived directly from some food industrial environment, without major intermediate steps.

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