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Research Article

Occurrence of 20S RNA and 23S RNA replicons in industrial yeast strains and their variation under nutritional stress conditions

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Abstract

We have characterized industrial yeast strains used in the brewing, baking, and wine-making industries for the presence or absence of cytoplasmic single-stranded 20S and 23S RNAs. Furthermore, the variation of intracellular concentrations of these replicons in brewing and laboratory strains under nutritional stress conditions was determined. Our results show a correlation between the relative abundance of these replicons and exposure of yeast to nutritionally stressful conditions, indicating that these RNAs could be employed as molecular probes to evaluate the exposure of $20S^+$ and/or $23S^+$ yeast strains to stress situations during industrial manipulation. During this study, several $20S^-23S^+$ *Saccharomyces cerevisiae* strains were isolated and identified. This is the first time that a yeast strain containing only 23S RNA has been reported, demonstrating that 20S RNA is not required for 23S RNA replication. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: ***Saccharomyces cerevisiae*; brewing yeast; double-stranded RNA virus; 20S RNA; 23S RNA; stress**

Introduction

Stress conditions in yeast cells may be defined as those environmental factors that cause a reduction of growth rate because they threaten cell survival (see review in Hohmann and Mager, 1997). In general, yeast cells exposed to such conditions display a rapid molecular response characterized by a general inhibition in gene expression and protein synthesis, enhanced synthesis of a limited set of proteins (heat-shock proteins) and accumulation of a few RNAs of unknown function (Estruch, 2000; Ruis and Schüller, 1995). In *Saccharomyces cerevisiae*, a temperature shift from 30°C to 37°C induces the synthesis of some double-stranded RNAs, known as W and T, in strains carrying such species (Wesolowski and Wickner, 1984).

S. cerevisiae harbours several autoreplicative viral RNA systems (reviewed in Esteban *et al*., 1993; Wickner, 1992, 1996). Wesolowski and Wickner (1984) identified and characterized two non-encapsidated double-stranded RNA (dsRNA) species, W (2.5 kb) and T (2.9 kb). Both W and T are linear RNAs (Rodriguez-Cousiño and Esteban, 1992) that are normally present in relative low copy number and are cytoplasmically transmitted. W and T also exist as single-stranded RNA (ssRNA) forms, 20S RNA (Kadowaki and Halvorson, 1971a; Matsumoto and Wickner, 1991) and 23S RNA (Esteban *et al*., 1992) respectively, although the functional relationship between the single and double-stranded forms is not known.

Synthesis of both single and double-stranded forms is induced by exposing the cells to stressful conditions, such as heat shock or nutritional starvation (Wesolowski and Wickner, 1984; Matsumoto *et al*., 1990). However, the role of these RNAs in the stress response and the regulation of their production are not understood. The 20S (W) RNA and 23S (T) RNA molecules have been cloned and

sequenced (Esteban *et al*., 1992; Matsumoto and Wickner, 1991; Rodriguez-Cousiño *et al*., 1991). Each element was found to contain a long open reading frame that appears to encode an RNA-dependent RNA polymerase with homology to those from RNA viruses (Esteban *et al*., 1994; García-Cuellar *et al*., 1995).

The variations of intracellular concentration of 20S (W) RNA and 23S (T) RNA have only been studied in laboratory strains. Some strains have been found to contain only 20S (W) RNA, i.e. $20S^+/23S^-$, while other strains have been found to contain both 20S (W) RNA and 23S (T) RNA, i.e. $20S^+/23S^+$. This shows that 23S (T) RNA is not required for replication of 20S (W) RNA. Strains containing only 23S (T) RNA, i.e. $20S^-/23S^+$, have not been reported previously.

We have undertaken the molecular characterization of 20S and 23S RNAs in industrial yeast strains from several fermentation industries, mainly the brewing industry. This is the first report of these RNA species in non-laboratory yeast strains. The increase in abundance of these RNAs in industrial strains under nutritional stress conditions, as well as their non-Mendelian inheritance, are reported. Our results demonstrate that 20S and 23S RNAs can be used to measure the stress response of yeast in industrial applications, and that there are some differences in the kinetics of accumulation of such RNA species in brewing and laboratory yeast strains.

Materials and methods

Strains, culture media and microbiological techniques

Industrial yeast strains were obtained from several bakeries, breweries and wineries, as well as the following culture collections: American Type Culture Collection (ATCC), Colección Española de Cultivos Tipo (CECT), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Mycotheque de l'Université Catholique de Louvain (MUCL), National Collection of Yeast Cultures (NCYC) and Valtion Teknillinen Tutkimuskeskus Collection of Industrial Microorganisms (VTT). *Saccharomyces diastaticus* (old nomenclature still in use in the brewing industry that describes *S. cerevisiae* strains that can grow on starch as the sole carbon source; Barnett, 1992) and *Saccharomyces* wild yeast strains were isolated as contaminants from breweries using standard methodologies (European Brewery Convention, 1987; American Society of Brewing Chemists, 1992). We have only worked with *Saccharomyces* isolates, since those RNAs have only been described in this genus. A summary of all yeast strains assayed is presented in Table 1.

Laboratory strains 37-4C (*MAT***a** L-0 $20S^+$ $23S^+$) (Wesolowski and Wickner, 1984), RE458 (*MATα* L–A–0 $20S^+$ $23S^-$) (Rodriguez-Cousiño *et al*., 1991), and AN33 (*MATα arg1 thr1* L-A-E $20S^-$ $23S^-$) (Garvik and Haber, 1978) were used as controls for 20S RNA and 23S RNA.

Yeast strains were normally grown at 30°C in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose). Starvation and sporulation medium contained only 1% potassium acetate. General techniques of yeast genetics, sporulation and dissection of tetrads were performed as described by Sherman *et al*. (1986).

Molecular cloning techniques

All techniques for molecular cloning were performed according to standard procedures (Sambrook *et al*., 1989).

Plasmids

pW1 (Ribas and Wickner, 1996) and pT27 (Esteban *et al*., 1992) (kindly provided by Professor R. Esteban, Instituto de Microbiología Bioquímica, University of Salamanca, Spain) contain cDNA fragments from 20S (W) RNA (1357 bp insert) and 23S (T) RNA (956 bp insert) respectively, and were used to develop probes for the detection of these RNAs.

p25S was used to obtain probes against 25S rRNA, in order to normalize the total RNA values in hybridization experiments. This plasmid was constructed as follows: (a) genomic DNA from an industrial brewing yeast strain was isolated as previously described (Cryer *et al*., 1975); (b) a 1537 bp fragment from the sequence of 25S rRNA was amplified by PCR (Primers: 5′-GTTCATCTA GACAGCCGGACGG-3′ and 5′-CCCACTAAAG GATCGATAGGCC-3′). Amplification conditions for PCR were as follows: 1 min at 95°C, 2 min at 44°C, and 3 min at 72°C for 30 cycles in a Perkin Elmer 2400 system (Norwalk, CA); (c) the PCR product was digested with *Xba*I and *Eco*RI, and cloned into pBluescript KS^+ (Stratagene, La Jolla, CA).

Table 1. Summary of yeast strains analysed for the presence of 20S RNA and 23S RNA

Yeast strains	$20S^{-}23S^{-}$	$20S^{+}23S^{-}$	$20S^{+}23S^{+}$	$20S^{-}23S^{+}$	Total
Brewing yeast from culture collections	20	7	3	0	30
Industrially used brewing yeast	30	7	0	0	37
S. diastaticus from culture collections	6	4	2	0	12
S. diastaticus isolated from breweries	12	6	8	4	30
S. cerevisiae wild yeast (other than *S. diastaticus*) isolated from breweries	14	0	2	0	16
Baking yeast	6	2	0	0	8
Wine-making yeast	25	1	0	0	26
Cider-making yeast	1	0	0	0	1
Total	114	27	15	4	160

RNA preparation

RNA samples for Northern analysis were prepared essentially as described by Rodriguez-Cousiño *et al.* (1991). To analyse the variation of 20S and 23S RNAs during starvation, RNA samples were obtained by phenol extraction with glass beads, as described by Sherman *et al.* (1986). RNA samples were further purified using RNeasy columns (Qiagen, Gilden, Germany), and quantified by measuring the absorbance at 260 nm.

Northern blot analysis

Northern hybridization analysis was performed basically as described by Fujimura *et al.* (1990). Samples containing 1 μg total RNA were separated on 1.2% agarose gels in 1 × TAE, transferred onto nylon membranes, and UV linked. Single-strand RNA-specific probes for hybridization were synthesized using the TransProbeT kit (Pharmacia, Uppsala, Sweden), γ-^{32}P UTP (400 Ci/mmol), and T3 polymerase, following the supplier's recommendations.

Kinetic studies on the variation of the intracellular concentration of 20S RNA and 23S RNA under starvation conditions

Yeast strains 37-4C and NCYC 1001 were grown in 100 ml YPD medium at 30°C for 48 h and transferred to sporulation medium. Five ml samples were collected at different times and stored at −80°C until being processed. After 60 h under starvation conditions, the remaining cells were transferred back to the same volume of YPD medium, and 5 ml samples were collected during an additional 48 h period. Dot blot analysis was performed with several dilutions in duplicate on sterilized nylon membranes.

Hybridization assays were done with 20S RNA or 23S RNA probes as described above. Hybridization signals were quantified using an Instantimager (Instantimager Electronic Autoradiography, Packard Instrument Company Inc, CT, USA). Probes were removed by boiling for 20 min with 0.01% SSPE plus 0.5% SDS prior the rehybridization of the membranes with the 25S rRNA probe as a control for total RNA loading.

Results

Screening for the presence of 20S RNA and 23S RNA in industrial yeast strains

A total of 160 yeast strains were analysed for the presence of 20S and 23S RNAs; 42 strains were obtained from culture collections, and the remaining 118 were supplied by, or isolated from, fermentation industries. The results are summarized in Table 1. Strains from culture collections containing 20S RNA and/or 23S RNA are described in Table 2. Figure 1 shows the Northern analysis of several representative yeast strains.

Among the 102 industrial strains analysed, approximately 20% contained one or both ssRNAs. The percentage of brewing strains from culture collections (33.3%) that contained 20S RNA was higher than that of currently employed industrial strains (18.9%). Only one out of 25 analysed wine strains (CECT 1329) contained 20S RNA. The results for wild yeast strains isolated from brewing fermentations depend on whether or not the strain belongs to the already abolished species of *S. diastaticus*. A high

Table 2. Yeast strains from culture collections that have been found to contain 20S RNA and 23S RNA

Strain	Description	20S RNA	23S RNA
CECT 1329	Wine strain from Jerez de la Frontera	+	–
CECT 10879	*S. diastaticus* isolated during this work	+	+
CECT 10880	*S. diastaticus* isolated during this work	–	+
CECT 10882	*S. diastaticus* isolated during this work	+	–
DSM 1848	Brewing yeast (lager)	+	–
MUCL 20463	Brewing yeast (ale)	+	+
NCYC 447	*S. diastaticus* strain from draught beer	+	–
NCYC 452	Brewing yeast (lager)	+	+
NCYC 625	*S. diastaticus* used in protoplast fusion studies (R.B. Gilliland, 1960)	+	–
NCYC 713	*S. diastaticus* from draught beer	+	+
NCYC 757	Brewing yeast (Coutts *et al.*, 1972)	+	–
NCYC 963	Brewing yeast (ale)	+	–
NCYC 990	*S. diastaticus* from Chivas Bros Ltd	+	–
NCYC 991	*S. diastaticus* from Chivas Bros Ltd	+	–
NCYC 1001	Brewing yeast (ale)	+	+
NCYC 1022	Brewing yeast (ale)	+	–
NCYC 1269	Brewing yeast (lager)	+	–
NCYC 1309	Brewing yeast (ale)	+	–
NCYC 1322	Brewing yeast (lager)	+	–
VTT-C-70060	*S. diastaticus* from beer	+	+

proportion of the *S. diastaticus* isolates from brewing samples (60%) and from culture collections (50%) were positive for one or the other ssRNA. However, only two of 14 other wild yeast strains analysed gave a positive result (Table 2). We have also isolated and identified for the first time four *S. diastaticus* strains that are $20S^-$ and $23S^+$.

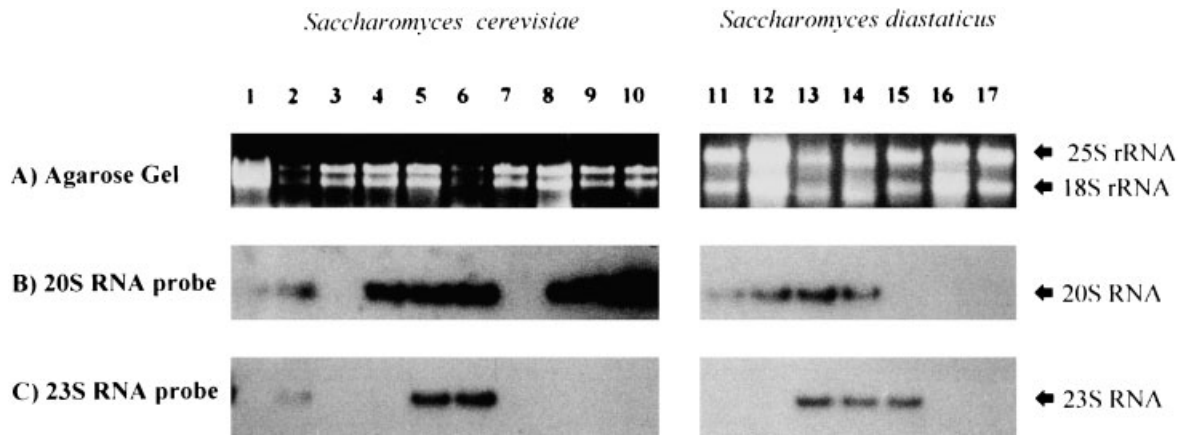

Figure 1. Northern hybridization analysis of industrial (*S. cerevisiae*, lanes 1–10) and wild yeast strains (*S. diastaticus*, lanes 11–17) with 20S RNA and 23S RNA probes, in order to analyse for the presence of such RNA species in the cells. (A) Agarose gel electrophoresis prior to RNA transfer to a nylon membrane. The rRNA bands shown on the figure were used as controls of RNA loading. (B) Northern hybridization with 20S RNA probe. (C) Northern hybridization with 23S RNA probe. Lane 1, RE458; lane 2, 37-4C; lane 3, AN33; lane 4, NCYC 963; lane 5, NCYC 452; lane 6, NCYC 1001; lane 7, commercial lager yeast strain; lane 8, DGC4; lane 9, CECT 1329; lane 10, commercial baking yeast strain; lane 11, NCYC 447; lane 12, CECT 10882; lane 13, NCYC 713; lane 14, CECT 10879; lane 15, CECT 10880; lane 16, CECT 10881; lane 17, VTT-C-68059

Table 3. Meiotic segregation of 20S RNA and 23S RNA in brewing yeast

Strain	Viable: non-viable spores	Number of tetrads analysed	$20S^+23S^+$: $20S^-23S^-$ [a]
NCYC 713	4:0	7	4:0 (7)
	3:1	2	3:0 (2)
NCYC 1001	4:0	7	4:0 (7)
	3:1	1	3:0 (1)
	2:2	3	2:0 (3)
NCYC 1001-1D	4:0	5	4:0 (5)
	3:1	2	3:0 (2)

[a]Numbers in parenthesis represent the number of analysed tetrads that showed such a segregation pattern.

Sporulation and segregation pattern for 20S RNA and 23S RNA in brewing yeast strains

To determine whether 20S RNA and 23S RNA were also cytoplasmically inherited in industrial and wild yeast strains, we sporulated eighteen $20S^+$ and $23S^+$ strains. Only two of them, NCYC 1001 (ale brewing yeast) and NCYC 713 (*S. diastaticus*), were able to sporulate. The results obtained are described in Table 3. All the meiotic segregants obtained showed the same parental phenotype. Some segregants from NCYC 1001 (NCYC 1001-1D) were selected and put under sporulation conditions again in order to analyse for the presence of these ssRNAs on the second generation of meiotic products. Once more, they demonstrated a $4^+:0^-$ segregation pattern for both RNAs. These results confirm that both RNA species are cytoplasmically transmitted in brewing and *S. diastaticus* strains.

20S RNA and 23S RNA as molecular probes for measurement of nutritional stress

We have shown that both 20S and 23S RNAs can be present in brewing yeast strains. It was still unclear, however, whether or not these RNAs were also increased under stress conditions. We approached this issue by comparing the variation of the ratio of intracellular concentration of each ssRNA to 25S ribosomal RNA (rRNA) when the yeast cells were placed in sporulation medium (i.e. nitrogen starvation or nutritional stress conditions). Figure 2 shows the results obtained, expressed as the variation of the ssRNA/25S rRNA ratio over time.

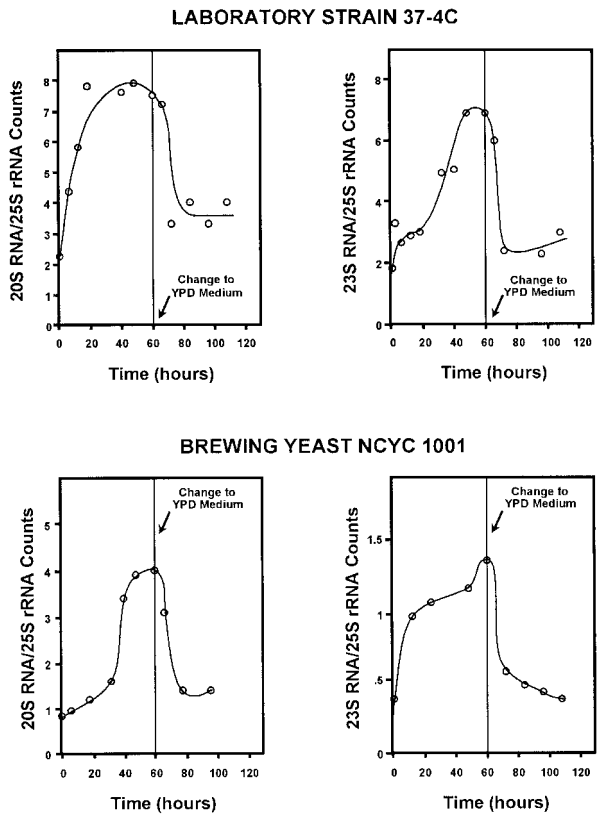

Figure 2. Variation of 20S RNA and 23S RNA under starvation conditions. RNA samples were taken from cultures of 37-4C (laboratory strain) and NCYC 1001 (industrial brewing strain) at different times, and the amount each ssRNA species was measured as described in Materials and methods. The graphics show the ratio ssRNA:25S rRNA over time. The amount of each RNA was measured as the number of counts after radioactive dot blot hybridization of 1 μg total RNA for each sample analysed. The vertical line shows the time when the cells were transferred from starvation (1% potassium acetate) to rich medium (YPD)

The same pattern was seen in both laboratory and industrial strains. This ratio starts increasing when the cells are put into sporulation medium, reaches a maximum, and decreases rapidly when the cells are returned to rich medium. However, there are some differences between the behaviour of the brewing yeast and the laboratory strain. First of all, such induction reaches higher ratios for both ssRNAs in the laboratory strain. The time needed to reach the maximum is shorter for the laboratory strain (18 h) than for the brewing yeast (48 h) in the case of 20S. Basal levels of both ssRNA are lower in the brewing yeast (the yield of ssRNA purified

from an equal number of cells is smaller in industrial strains), although the ratio rises approximately the same in both strains: approximately four-fold for 20S RNA and 3.5-fold for 23S RNA. It remains unclear whether this is a general trend or whether there is dependence on the genetic background of the strain, or on other factors such as availability of intracellular reserves. Moreover, different yeast stress situations under various industrial conditions may have a different impact on these ratios. The relative increase in the intracellular content of 20S RNA and 23S RNA is reversible for both industrial and laboratory strains.

Discussion

Current industrial practices may produce variations in yeast physiology, viability and fermentation performance, and may result in contamination with wild yeast or other strains used in the same facility. Consequently, it is essential to design a reliable and rapid test to assess the suitability for the industrial use of a specific batch of yeast (Boulton *et al.*, 1991).

20S RNA and 23S RNA are autonomous replicons present in yeast cells whose intracellular concentration is greatly amplified under heat shock or nutritional starvation conditions (Matsumoto *et al.*, 1990; Estaban *et al.*, 1992). Thus, both ssRNA species could be used as molecular probes to monitor stress conditions in yeast used for industrial purposes. However, all previous work related to 20S RNA and 23S RNA had been done in laboratory strains; therefore the occurrence and behaviour of these ssRNAs in industrial yeast strains was not known. Thus, we first tested industrial and wild yeast strains for the presence or absence of such ssRNAs. We analysed 160 yeast strains from culture collections or industry isolates (72 were used industrially and 46 were putative contaminant strains, mostly *S. diastaticus*). Contrary to what was previously reported for laboratory strains, most industrial yeast strains used in this study do not contain 20S RNA, while most *S. diastaticus* strains do. However, percentage results obtained from industrial yeast strains should be interpreted with caution, due to the lack of information regarding their origin. *S. diastaticus* is one of the most threatening contaminants in the brewing industry because of the undesirable off-flavours it produces. This increased occurrence of 20S and 23S RNAs in *S. diastaticus* may provide a means of detecting contamination of brewing yeast prior to its use in a fermentation, provided that the industrial yeast strain in use is $20S^-$.

In our study, we were able to isolate and characterize 15 additional $20S^+$ $23S^+$ strains, which had only been reported previously by Wesolowski and Wickner (1984). We also found four *S. diastaticus* strains that were $20S^-23S^+$. This is the first time that a yeast strain containing 23S RNA without 20S RNA has been described, proving that the 20S species is not necessary for 23S RNA replication, and making a $20S^-23S^+$ strain available for further studies.

Although there was not a correlation between the presence of such ssRNAs and common fermentation characteristics of the strains, a further characterization of industrial yeast strains or a protocol able to introduce these replicons in other strains might help to establish whether there is any phenotype associated with the presence of these RNAs that could be of interest to fermentation industries.

20S RNA was first described as being induced by the starvation conditions required for sporulation (Kadowaki and Halvorson, 1971a, b). Industrial yeast strains tend to be unable to sporulate because they are typically hemizygous and aneuploid. Only two among 18 $20S^+/23S^+$ industrial and wild yeast strains analysed were able to sporulate. This fact confirms that such ssRNA species are very unlikely to be involved in sporulation processes, but rather induced under the same conditions in which sporulation takes place, as suggested by Garvik and Haber (1978). Furthermore, they proved to be autonomous replicons, as in laboratory strains, since they are also cytoplasmically inherited in industrial strains.

Measuring the relative concentration of such RNA species under starvation conditions over time allowed us to analyse the accumulation of 20S RNA and 23S RNA in both laboratory and industrial yeast strains. Previous studies have shown that 20S RNA production is highly induced when laboratory yeast cultures are under stress conditions (Kadowaki and Halvorson, 1971a; Garvik and Haber, 1978; López *et al.*, 1997). We observed that the same phenomenon happens for 23S RNA, and that it is a general phenomenon for laboratory and industrial strains.

In conclusion, we have demonstrated the occurrence of 20S RNA and 23S RNA in wild and industrial yeasts, and their increase when cells have been exposed to nutritional stress conditions. Our results indicate that such ssRNA species could be used as indicators of yeast stress conditions in industrial processes, as well as molecular markers to detect contamination from wild yeast strains, more specifically from *S. diastaticus* strains. Rapid and quantitative techniques based on RT–PCR methodologies need to be developed in order to successfully apply them in routine quality control programs at the industrial level.

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