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Incidence of *SUC-RTM* telomeric repeated genes in brewing and wild wine strains of *Saccharomyces*

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Abstract When over-expressed, *RTM* yeast genes confer resistance to the toxicity of molasses. They are found in distiller's and baker's industrial yeasts in multiple copies, scattered on the telomeres and physically linked to the telomeric *SUC* genes. Because these genes are absent from some laboratory strains, we explored the genomes of other industrial yeasts (brewing strains) and wine wild strains. A collection of 47 wine yeast strains (*S. cerevisiae* and *S. bayanus*) and 15 brewing strains, lager, ale and possible ancestors (*S. monacensis*, *S. paradoxus* and *S. carlsbergensis)* were screened for the presence of *RTM* genes. Only three wine strains and all brewing strains proved to contain *RTM* sequences in different copy numbers. PCR and chromosome blotting confirm the presence of *SUC* sequences in tandem with *RTM*. Moreover, analysis of the entire *S. cerevisiae* genome sequence shows that three other, non-telomeric, genes related to *RTM* are scattered on different chromosomes.

Key words Wine yeasts · Brewing yeasts · *RTM* genes · *SUC* genes

Introduction

Chromosome ends are characterised in yeast by their high variability (Louis 1995). Gene families like *SUC* (Carlson and Botstein 1983), *RTM* (Ness and Aigle 1995), *MEL* (Naumov et al. 1996), *STA* (Pretorius and Marmur 1988), *MAL* (Charron et al. 1989) and *PHO* (Venter and Hörz 1989) can be present or absent on different chromosome ends. Because all these genes, except *RTM*, are directly involved in carbon- or phosphate-source utilisation, this can be seen as a genetic adaptation to different substrates by yeast.

Industrial yeast fermentation of sucrose (contained in molasses) produces most of the ethanol and yeast biomass. Beet molasses can be toxic for industrial yeasts, but different strains have various levels of resistance. From these observations, Ness and Aigle (1995) isolated the *RTM1* gene leading to resistance to toxic molasses, by using an over-expression strategy in a laboratory strain. This gene, encoding a potential hydrophobic 34-kDa protein, turned out to be a member of a highly conserved gene family. For distiller's and baker's yeasts, *RTM* genes are present in multicopies dispersed at the ends of chromosomes and are linked to *SUC* telomeric genes. The presence of multiple *RTM* genes in these strains may result from adaptation to growth in toxic molasses. In parallel, the presence of multicopies of *SUC* genes encoding invertase (Carlson and Botstein 1983) may also result from an adaptation to growth on sucrose, which is almost the only carbon source in molasses. It was then relevant to analyse the status of *SUC* and *RTM* genes in different yeast strains, whether industrial or not, that were not traditionally grown on sucrose as a carbon source.

Wine yeast strains usually possess only one copy of the *SUC* gene, *SUC2*, located far from the telomeres (Bidenne et al. 1992). Grape-must sugars are glucose and fructose instead of the sucrose found in molasses. In this work we test the presence or absence of the *RTM* genes in a collection of wild wine strains of *Saccharomyces*, composed of 30 commercial strains which are generally industrially cultivated on molasses, and 17 indigenous strains which have never been cultivated on sucrose. All these strains are thought to be a direct isolation of indigenous yeasts from spontaneous fermentation, without further crosses.

Brewing yeast strains are grown mainly on maltose as a carbon source. Their taxonomical status is not yet clear,

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but two main families are used: lager strains (*S. carlsbergensis*) which are hybrids between *S. cerevisiae* and another species, probably *S. monacensis* (Hansen and Kielland-Brandt 1984), and ale strains which are *S. cerevisiae*. We also checked the status of *SUC-RTM* tandem structure in 15 different strains.

Finally, in an attempt to enlarge our understanding of the entire system, and because of the presence of an isolated *SUC2* gene on Chromosome IX, we performed a search of the total yeast genome sequence, looking for other genes related to the *RTM* family.

Materials and methods

Yeast strains. The laboratory strains of *S. cerevisiae* used (Table 1) were FL100 (ATCC 28383) and X2180-1A (Yeast Genetic Stock Center, Bekerley, USA). A collection of 47 *Saccharomyces* wine strains was composed of: (1) 30 commercial strains (these strains were traditionally isolated from spontaneous grape-must alcoholic fermentations), (2) 17 indigenous strains isolated from must-fermentations in the Bordeaux producing area (Laboratoire d'Œnologie Générale, Faculté d'Œnologie, Bordeaux, France). In the wine collection, 44 strains belonged to *S. cerevisiae* and three to *S. bayanus* according to a PCR/RFLP analysis of the *MET2* gene (Hansen and Kielland 1994; Masneuf et al. 1996). A collection of 15 *Saccharomyces* brewing strains was composed of: (1) five lager strains, (2) four ale *S. cerevisiae* strains, (3) three "weissenbier" strains, and (4) three possible ancestors, *S. pastorianus*, *S. monacensis* and *S. carlsbergensis* (probably a hybrid between *S. monacensis* and *S. cerevisiae*). All strains were grown in YPD medium (10 g/l yeast extract, 10 g/l peptone, 20 g/l glucose).

DNA amplification tests. Amplifications were performed on genomic DNA or directly on cells grown on YPD plates. In the latter case, stationary phase yeast clones were picked up with a straight platinum wire, dispersed in 10 µl of sterile water and heated to 100°C for 10 min prior to amplification. Primers used to detect *RTM* genes were RTM51 [5′TCAAATGACTCTAGTGGCTCT3′; nucleotides 667–687 from Ness and Aigle (1995)] and RTM31 (5′ACACCTCATAACATGCAGTAG3′; nucleotides 1087–1107). To detect *SUC-RTM* linkage, the primers used were RTM31 and SUC32 (5′ATTGACAAGTTCCAAGTAAGG3′; nucleotides 2369–2389 from *SUC1* sequence, EMBL accession number X07570). See Fig. 1 for primer location. PCR reactions were performed in a thermal "Mini Cycler ™" (M. J. Research, Watertown, USA) using the following solution: 50-µl reaction mixtures were prepared with 100 pmoles of each primer, 10 mM Tris-HCl pH 9, 0.1% triton X-100, 1.5 mM $MgCl_2$, 0.2 mg/ml BSA, 200 µM each dATP, dCTP, dGTP, dTTP, 1.5 U of *Taq* DNA polymerase (Appligene, Illkirch, France) and 10 µl of heated cells or 100 ng of DNA. For the *RTM* test, DNA from heated cells was amplified for 30 cycles with the following programme: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. For the linkage test, genomic DNA was amplified for 25 cycles with the following programme: 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min. For analysis, 5 µl of the amplification product were separated on a 0.8% agarose gel.

Resistance to molasses. Drops of a light yeast suspension were loaded onto plates to perform the sensitivity test to molasses. The molasses media were composed of 10, 20, 30 or 40% (w/v) molasses, 5 g/l YNB (Difco) in 0.1 M pH 6 succinate buffer. Growth was monitored after 2 days at 30°C.

Contour-clamped homogenous electric field (CHEF) electrophoresis. Preparation of chromosomal DNA was performed as described by Carle and Olson (1985). An electrophoretic apparatus CHEF-DR III (BIO-RAD Laboratories, USA) was used to separate the yeast

Table 1 List of laboratory, wine and brewing yeast strains used in this study

Number	Strain	Species	Source	Origin
	FL100	*S. cerevisiae*	ATCC 28383	
	X2180-1A	*S. cerevisiae*	Yeast Genetic Stock Center	
1	522DAVIS	*S. cerevisiae*	Commercial	Wine
2	F5	*S. cerevisiae*	Commercial	Wine
3	71B	*S. cerevisiae*	Commercial	Wine
4	MON86	*S. cerevisiae*	Commercial	Wine
5	RC212	*S. cerevisiae*	Commercial	Wine
6	ICVD254	*S. cerevisiae*	Commercial	Wine
7, 9	7303	*S. cerevisiae*	Commercial	Wine
8	SM102	*S. cerevisiae*	Commercial	Wine
10	C94	*S. cerevisiae*	Commercial	Wine
11	Wädenswil27	*S. cerevisiae*	Commercial	Wine
12	L-2056	*S. cerevisiae*	Commercial	Wine
13	EG8	*S. cerevisiae*	Commercial	Wine
14	UP3OY5	*S. cerevisiae*	Commercial	Wine
15	522DAVIS KL	*S. cerevisiae*	Commercial	Wine
16	GLO7447	*S. cerevisiae*	Commercial	Wine
17	L2868+L2872	*S. cerevisiae*	Commercial	Wine
18	L1597	*S. cerevisiae*	Commercial	Wine
19	WET136	*S. cerevisiae*	Commercial	Wine
20	VL3C	*S. cerevisiae*	Commercial	Wine
21	VL1	*S. cerevisiae*	Commercial	Wine
22	BO213	*S. cerevisiae*	Commercial	Wine
23	67CJ	*S. cerevisiae*	Commercial	Wine
24	R2	*S. cerevisiae*	Commercial	Wine
25	F10	*S. cerevisiae*	Commercial	Wine
26	AWRI796	*S. cerevisiae*	Commercial	Wine
27	B	*S. cerevisiae*	Commercial	Wine
28	AC-	*S. cerevisiae*	Commercial	Wine
29	FZ′IIB4	*S. cerevisiae*	Indigenous	Wine
30	FZ′IIB6	*S. cerevisiae*	Indigenous	Wine
31	SO24	*S. cerevisiae*	Indigenous	Wine
32	SO28	*S. cerevisiae*	Indigenous	Wine
33	YIC8	*S. cerevisiae*	Indigenous	Wine
34	17e4	*S. cerevisiae*	Indigenous	Wine
35	Sc58	*S. cerevisiae*	Indigenous	Wine
36	ChIb20	*S. cerevisiae*	Indigenous	Wine
37	ChIb25	*S. cerevisiae*	Indigenous	Wine
38	Tb3IIb4	*S. cerevisiae*	Indigenous	Wine
39	7013	*S. cerevisiae*	Commercial	Wine
40	St Georges	*S. cerevisiae*	Commercial	Wine
41	S6U	*S. uvarum*	Commercial	Wine
42	VS6	*S. bayanus*	Indigenous	Wine
43	Y′Ic1	*S. cerevisiae*	Indigenous	Wine
44	Y′Ic2	*S. cerevisiae*	Indigenous	Wine
45	Tb3IVc28	*S. bayanus*	Indigenous	Wine
46	LgIb23	*S. cerevisiae*	Indigenous	Wine
47	LgIb28	*S. cerevisiae*	Indigenous	Wine
48	K9	*S. carlsbergensis*	Lager	Brewing
49	C16	*S. carlsbergensis*	Lager	Brewing
50	H	*S. carlsbergensis*	Lager	Brewing
51	TbC1	*S. carlsbergensis*	Lager	Brewing
52	CbC2	*S. carlsbergensis*	Lager	Brewing
53	D	*S. cerevisiae*	Ale	Brewing
54	T	*S. cerevisiae*	Ale	Brewing
55	C	*S. cerevisiae*	Ale	Brewing
56	J	*S. cerevisiae*	Ale	Brewing
57	68C1	*S. cerevisiae*	Weissenbier	Brewing
58	296C1	*S. cerevisiae*	Weissenbier	Brewing
59	297C1	*S. cerevisiae*	Weissenbier	Brewing
60	Mo	*S. monacensis*	Ancestors	
61	Pa	*S. pastorianus*	Ancestors	
62	Ca	*S. carlsbergensis*	Ancestors	

chromosomes. Electrophoresis was carried out at 6 V/cm with a buffer temperature maintained to 14°C, for 15 h with a switching time of 60 s, and 9 h with a switching time of 90 s. The running buffer was 0.5×TBE (45 mM Tris; 45 mM borate; 1 mM EDTA; pH 8.3). The gel was made with 1% agarose. A standard set of *S. cerevisiae* YNN295 chromosomes was used and *S. cerevisiae* X2180 and FL100 were employed as reference strains.

Chromosome-blot analysis. Chromosomal DNA separated by CHEF was treated and transferred to nylon filters as described by Maniatis et al. (1982). The *RTM1* probe was a 0.9-kpb *Eco*RI-*Bam*HI fragment isolated from p1K (Ness and Aigle 1995) covering most of the ORF. The *SUC2* probe was a 1.6-kpb fragment generated by amplification in vitro with the SUC2G (5′ATGCTTTTGCAAGCTTTCC3′) and SUC2D (5′ATTTTACTTCCCTTACTTGG3′) primers. The amplified DNA from the X2180 strain entirely covered the ORF. The probes were labelled with digoxigenin-11-dUTP using the non-radioactive DNA labelling and detection kit (Böhringer Mannheim, Mannheim, Germany). Hybridisation and chemiluminescent detection with CSPD were done as described in the kit.

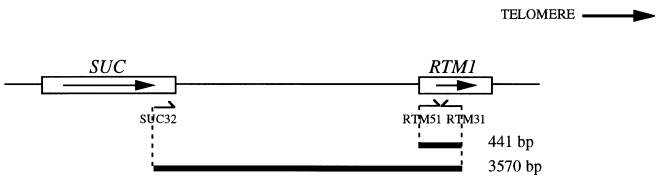

Fig. 1 Amplification strategy to detect *RTM* genes and *SUC-RTM* linkage in yeast. Amplification products are in *bold lines*

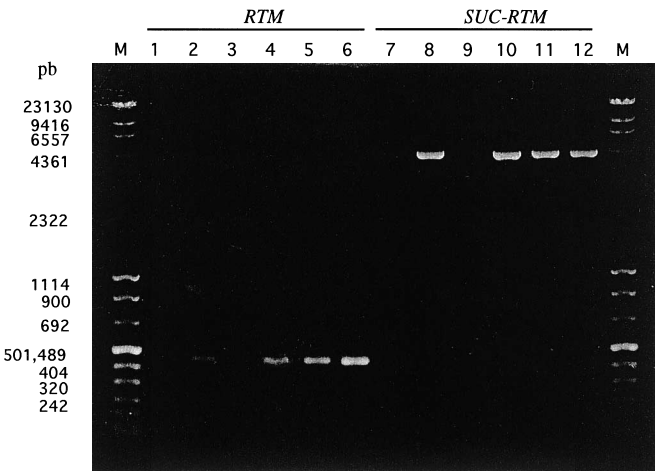

Fig. 2 Amplifications to detect *RTM* sequences and *SUC-RTM* linkage in yeasts. Lanes *1* to *6* show DNA amplifications using RTM51/RTM31 primers to detect *RTM* genes. Lanes *7* to *12* show DNA amplifications using SUC32/RTM31 primers to detect *SUC-RTM* linkage. Wells 1 and 7 are PCR negative controls. Control strains shown are FL100 positive strain (lanes *2* and *8*) and X2180-1A negative strain (lanes *3* and *9*); the three positive wine strains 3, 16 and 40 (lanes *4*, *5*, *6* and *10*, *11*, *12*). *M*, molecular markers

Results

Incidence of *RTM* genes in 47 wine yeasts

We tested the presence or absence of *RTM* genes in a group of 47 wine yeasts by amplification of a 441-bp DNA fragment (codon 2 to codon 148 of the *RTM1* gene, Fig. 1). We also included a positive strain, FL100, and a negative strain, X2180-1A (Ness and Aigle 1995). Only three commercial *S. cerevisiae* strains (numbers 3, 16 and 40) were positive (Fig. 2). One of these strains, number 3, was described by Bidenne et al. (1992) as possessing multiple *SUC* alleles. The origin of these three strains is not reliably known. In any case, *RTM* genes proved to be rare among our wild wine strain collection.

Incidence of *RTM* genes in 15 brewing yeasts

As described for the wine yeasts, we tested the presence or absence of *RTM* genes in a group of 15 brewing yeast strains. All the strains were positive, giving the same pattern as shown in Fig. 2 for positive wine strains.

RTM-SUC linkage

Using restriction analysis, Ness and Aigle (1995) showed that in FL100 the *RTM1* gene is linked to the *SUC7* gene (chromosome VIII). More generally, restriction analysis and chromoblot results suggested that all *RTM* genes were linked to *SUC* at the telomeres. The two genes are separated by about 3100 bp. Consequently, we tested the physical linkage between *RTM* and *SUC* in wine and brewing *RTM*-positive strains. This was first done by amplification using one primer (SUC32) at the end of *SUC* and another in *RTM* (RTM31) (Fig. 1). As shown in Fig. 2, as expected a 3570-bp DNA amplification fragment was obtained with FL100, and also with the three wine strains 3, 16 and 40 and every brewing strain (data not shown). X2180 was used as a negative control. These results confirmed the physical linkage between *RTM* and *SUC* in all strains described.

Chromosomal DNA analysis of control strains (FL100 and X2180), the three *RTM-SUC*-positive wine strains (numbers 3, 16 and 40), two *RTM-SUC*-negative wine strains (numbers 23 and 24), and 6 of the 15 all *RTM-SUC*-positive brewing strains (numbers 48, 53, 57, 60, 61 and 62) was done using CHEF electrophoresis and blotting (Fig. 3). Reference strains FL100 and X2180 gave the expected hybridisation patterns (Ness and Aigle 1995) with the *SUC2* and *RTM1* probes: two chromosomal bands for FL100 corresponding to *SUC2* (chromosome IX) and *SUC7* (chromosome VIII) and one corresponding to *RTM1* (chromosome VIII); one chromosomal band for X2180 corresponding to *SUC2*; and no hybridisation with the *RTM1* probe. All the strains tested carried the *SUC2* allele and, except the two *RTM*-negative wine strains, they all carried 2–6 supernumerary *SUC* genes. Despite strong heterogeneity between signal intensities, different exposures of the blots show that when the *RTM* genes were present they were always on the same chromosomes as the *SUC* genes, in accordance with the results obtained by PCR.

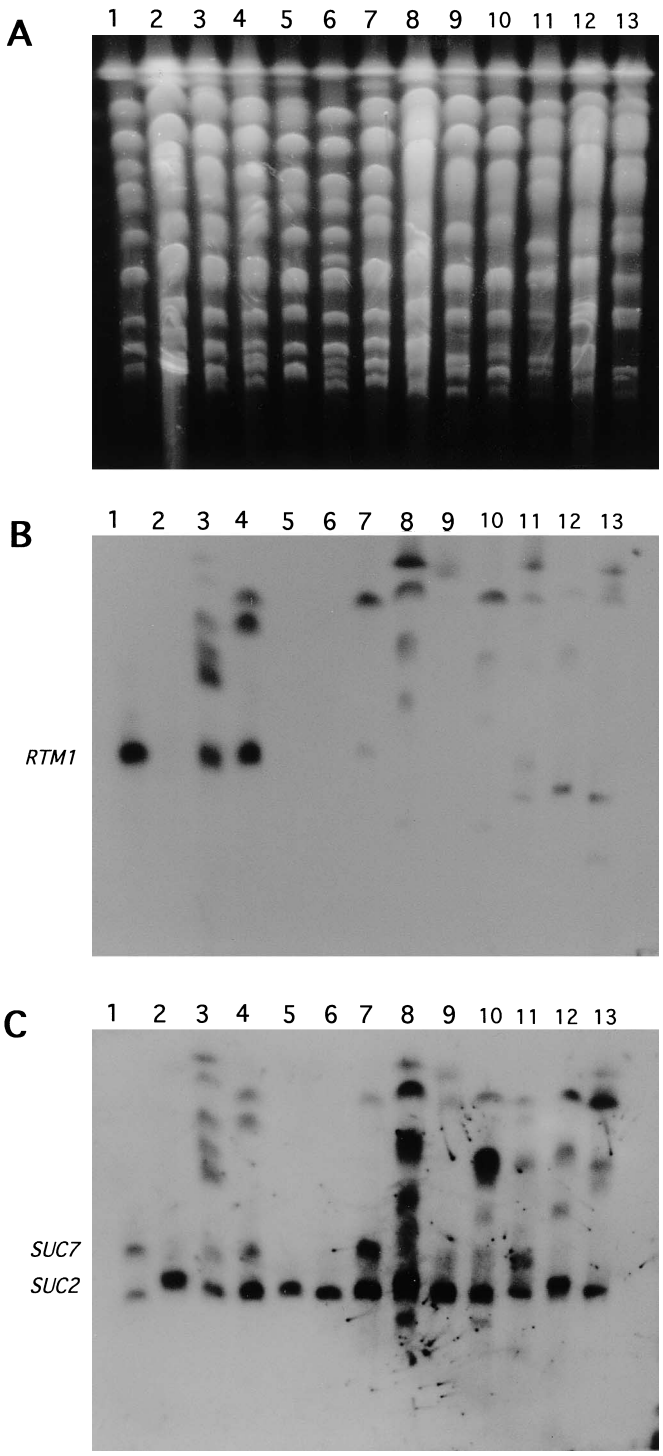

Fig. 3A–C Location, number and linkage of *RTM* and *SUC* genes in different strains of yeast. Hybridisations were done on chromosomal DNA (**A**) with the *RTM1* (**B**) and the *SUC2* (**C**) probes. Strains loaded were: (i) wine strains no 3 (lane *3*), no 16 (lane *4*), no 23 (lane *5*), no 24 (lane *6*) and no 40 (lane *7*). (ii) brewing strains no 48 (lane *8*), no 53 (lane *9*), no 57 (lane *10*), no 60 (lane *11*), no 61 (lane *12*) and no 62 (lane *13*). (iii) control strains FL100 (lane *1*) for *RTM1*, *SUC2* and *SUC7* genes and X2180 (lane *2*) a *RTM* negative strain for *SUC2* gene

Sensitivity of wine and brewing yeasts to molasses

Forty wine strains were tested for their ability to grow on plates containing molasses in increasing concentrations. Among them, 29, including the number 3 *RTM*-positive wine strain, were inhibited like the sensitive laboratory strains at a concentration of 20% molasses. The remaining 11, including two *RTM*-positive wine strains (numbers 16 and 40), still grew at this concentration. Only two strains grew at 30% molasses including the *RTM*-positive strain number 16. The 15 brewing strains were also tested. Only eight strains were still able to grow at 20% molasses and no strain grew at 30%. So no strict correlation was found between presence of *RTM* genes in strains and their resistance to molasses. Moreover, the growth was very heterogenous in all concentrations of molasses due to the very different vigour of the strains.

On one hand, the fact that *RTM*-positive strains were not all very resistant to toxic molasses can be related to *RTM* gene expression, which is not known. On the other hand, *RTM*-negative strains can offer resistance to toxic substances by mechanisms other than the *RTM*-encoded one. For wine strains, one *RTM*-positive strain out of three was very resistant to molasses, compared to one *RTM*-negative strain among 33.

Genome-sequence analysis

Apart from the results concerning the very closely related *RTM* genes dispersed with the *SUC* genes on the telomeres, no other unexpected PCR fragments or hydridisation patterns were detected in our study. This suggests that, contrary to the case of *SUC*, for which an internal *SUC2* is always present in all strains, (Carlson and Botstein 1987; Ness and Aigle 1995) no other *RTM* sequences exist in the yeast genome. Nevertheless, it is possible that related genes (paralogs, defined as non-identical but related genes inside the same genome) exist in yeast. We took advantage of the availability (e.g. SGD 1997 or MIPS 1997) of the whole sequence of the genome of the S288C strain to look for *RTM* paralogs. Based on amino-acid sequences, using the BLAST and FASTA programs, three sequences were found to have strong similarity to *RTM*. The closest is *YER185w* (identity 61%, chromosome V), of which nothing is known about its function. The second closest gene is *RTA1* (identity 40%, chromosome VII, Soustre et al. 1996), which has been identified in a screen for resistance to amino-cholesterol. The third, *YLR046c* (identity 25%, chromosome XII), the product of which is less related, presents only four potential transmembrane domains instead of seven for other proteins. No information is available on the function of *YLR046c*. All four genes share a typical low codon adaptation index, 0.093 for *RTM1*, 0.104 for *YER185w*, 0.118 for *RTA1* and 0.102 for *YLR046c* respectively. The fact that no one of these is linked to *SUC2* is noteworthy.

Discussion

These results point to several intriguing questions concerning the physiological function and genetic evolution of the *SUC-RTM* tandem unit.

(1) Physiologically, two elements have to be considered. First, the *SUC* genes encode invertase (Carlson et Botstein 1983). Amplification of this gene can be understood in the context of baker's and distiller's yeast strains, which are industrially grown on sucrose as a carbon source. In the case of the brewing yeasts the meaning of this specific amplification of these genes is not obvious since maltose is the substrate in the brewing process. Nevertheless, the presence of telomeric *SUC* genes in "ancestor" strains suggests that, at least in this case, the origin of amplification is perhaps not linked to industrial use but rather to the genetic origin of the strains. With regard to the wine yeasts, the absence of supernumerary *SUC* genes is in accordance with the absence of sucrose in grape juice. It is nevertheless relevant to note that sucrose is the universal sugar in plant sap. The second point to consider concerns the *RTM* genes. First identified as conferring resistance to an unidentified toxic element in molasses, the actual physiological function of the Rtmp is not known. We tested all strains described in this study on different concentrations of molasses, but the results were difficult to interpret. The presence of numerous *RTM* genes in brewing strains is, in this respect, not easy to understand, because brewing-wort is not known generally to be toxic. Considering the origin of molasses and wort, it is nevertheless possible that a common compound, originating from the heating of plant material in the presence of a high concentration of sugar, could be present in both industrial substrates. This would not be the case in grape juice, since it is used fresh. The fact that an *RTA1* paralog has also been isolated by its property to confer resistance to a toxic metabolite (amino-cholesterol, Soustre et al. 1996) suggests that the actual function of the family could be part of a specific de-toxification process (no cross resistance exists between the two systems, Soustre et al. 1996).

Another question is the genetic linkage between *SUC* and *RTM* genes. In the case of the *MAL* loci, the three linked *MAL* genes are necessary for maltose utilisation (Needleman et al. 1984). As strains devoid of *RTM* genes are nevertheless able to grow on sucrose (e.g. S288C), this gene is obviously not necessary for sucrose metabolism. Whenever we have tested *SUC-RTM* association by PCR and chromosome blotting, we always found an association between them, at least once in each strain. A tentative hypothesis is that *RTM* is involved in the handling of some toxic metabolite, biologically associated with sucrose in the plant material.

(2) Genetically, the simple idea that spreading of the *SUC-RTM* tandem has occurred under industrial conditions as a result of selective pressure (Ness and Aigle 1995) has to be revised in the light of these results. In fact the brewing-wort does not present parameters in accordance with this idea, i.e. sucrose and obvious toxicity. Another explanation could be found in the history of the industrial strains: baker's and distillary strains are possibly derived from brewing strains (Fould-Springer 1988). Additionally, the use of beet molasses (sucrose) as an industrial substrate for growing yeasts is recent (about 1935) (Fould-Springer 1988). The old process used malt as a substrate. Thus, a part of the answer could well be the serendipitous historical origin of the baker's yeast strains, fortunately followed by a good biological fitness relative to the use of molasses in this industry.

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