RTM1: A Member of a New Family of Telomeric Repeated Genes in Yeast

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ABSTRACT

We have isolated a new yeast gene called *RTM1* whose overexpression confers resistance to the toxicity of molasses. The *RTM1* gene encodes a hydrophobic 34-kD protein that contains seven potential transmembrane-spanning segments. Analysis of a series of industrial strains shows that the sequence is present in multiple copies and in variable locations in the genome. *RTM* loci are always physically associated with *SUC* telomeric loci. The *SUC-RTM* sequences are located between X and Y′ subtelomeric sequences at chromosome ends. Surprisingly *RTM* sequences are not detected in the laboratory strain X2180. The lack of this sequence is associated with the absence of any *SUC* telomeric gene previously described. This observation raises the question of the origin of this nonessential gene. The particular subtelomeric position might explain the *SUC-RTM* sequence amplification observed in the genome of yeasts used in industrial biomass or ethanol production with molasses as substrate. This *SUC-RTM* sequence dispersion seems to be a good example of genomic rearrangement playing a role in evolution and environmental adaptation in these industrial yeasts.

GENE overexpression or increase in the copy number of a gene is a common adaptative mechanism exploited by different organisms to increase their resistance to various toxic agents. A particularly well-studied example of the gene amplification processes involves the increased specific resistance to copper. Copper resistance in yeast is controlled by the *CUP1* locus. The level of resistance is proportional to the copy number of this gene, which can be found in up to 15 tandemly repeated copies (WELCH *et al.* 1983; KARIN *et al.* 1984).

Resistance to multiple cytotoxic compounds is another acquired property in many species from bacteria to man. The major molecular determinants mediating multidrug resistance are transport proteins driving the traffic of drugs and physiological substrates across the cell membrane, including the major facilitors superfamily (MARGER and SAIER 1993) and the ATP-binding cassette superfamily (ABC) (reviewed by HIGGINS 1992). The former family comprises the yeast aminotriazole-resistance determinant *ATR1* (KANAZAWA *et al.* 1988; GÖMPEL-KLEIN and BRENDEL 1990); the latter includes the yeast *STE6* pump for secretion of the mating pheromone **a** (KUCHLER *et al.* 1989; MCGRATH and VARSHAVSKY 1989). A common mechanism underlying multidrug resistance is overexpression of transport proteins, for example, *MDR1* in tumor cell lines (GOTTESMAN and PASTAN 1993) and *PDR5* in yeast (LEPPERT *et al.* 1990).

Other gene amplification events have been described in *Saccharomyces cerevisiae.* Three families of duplicated genes illustrate the genetic polymorphism of the yeast Saccharomyces and underline another gene amplification process. They comprise the *SUC, MAL* and *MEL* gene families, which encode enzymes necessary for the utilization of sucrose, maltose and melibiose, respectively. The *MEL* gene family of *S. cerevisiae* consists of ≥10 structural genes *MEL1–MEL10* (NAUMOV *et al.* 1990, 1991; TURAKAINEN *et al.* 1993); the *MAL* gene family comprises the five highly homologous *MAL1, 2, 3, 4* and *6* (CHARRON *et al.* 1989), and the *SUC* gene family includes the six loci *SUC1, 2, 3, 4, 5* and *7* (CARLSON and BOTSTEIN 1983; CARLSON *et al.* 1985). All of these genes (except *SUC2*) are located near chromosome ends; they are dispersed on different chromosomes but generally not all of them are present simultaneously. The particularity of the *SUC* telomeric genes is their specific location between the Y′ and X telomere-associated sequences. As has been suggested for the *SUC* genes and the Y′ and X subtelomeric sequences, the two other gene families may have resulted from translocation events involving the telomeres of different chromosomes (CARLSON *et al.* 1985; CHARRON *et al.* 1989; LOUIS and HABER 1990b; MICHELS *et al.* 1992; TURAKAINEN *et al.* 1993).

Studying some distiller's yeasts, we observed that some of the molasses used as substrate in industrial ethanol production confer growth inhibition. Previous studies (VON TRESSL *et al.* 1976; FIEDLER 1981; BRONN and FATTOHI 1988) tried to identify the toxic elements but have failed due to the complex composition of such media. As we have observed that strains exhibit various levels of resistance to toxic molasses, we attempted to isolate a gene conferring resistance to toxic molasses

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TABLE 1

Genotypes of the laboratory strains used in this study

Strain	Genotype	Sources
FL100	*MAT***a** wild type	ATCC 28383
FL100m	*MAT***a** *trp1 ura3*	F. LACROUTE
FL100m-2n	*MAT***a** *trp1 ura3* / *MATα trp1 ura3*	F. LACROUTE
FL100-Δ1[a]	*MAT***a** *trp1 ura3 rtm1-Δ1::URA3*	This study
X2180-1B	*MATα SUC2 mal mel GAL2 CUP1*	Yeast Genetic Stock Center
LG16-1A	*MAT***a** *ura3*	Our laboratory/CROUZET *et al.* (1991)
LG16-5A	*MATα ura3*	Our laboratory/CROUZET *et al.* (1991)
LG16-2n[b]	*MAT***a** *ura3* / *MATα ura3*	Our laboratory/CROUZET *et al.* (1991)

[a] Deletion of *RTM1* gene in the strain FL100-Δ1 was obtained by one-step gene replacement as described in MATERIALS AND METHODS.

[b] LG16-2n strain was sporulated to obtain LG16-1A and LG16-5A. LG16 strains are isogenic to X2180.

(*RTM*). By analogy with several other resistance genes, we looked for such a gene using a multicopy (overexpressing) genomic library. By this experimental procedure we isolated the *RTM1* gene that, overexpressed, confers a resistance phenotype against toxic molasses. We explored the genome of different strains and observed the genetic diversity of *RTM1* in distiller's yeasts. We describe that *RTM1* is a member of a new telomeric family of genes dispersed thoughout the yeast genome and physically associated with the *SUC* telomeric loci.

MATERIALS AND METHODS

Strains and media: The growth media used were YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose), YNB (6.7 g/l Yeast Nitrogen Base (Difco), 20 g/l glucose) and YPGE (10 g/l yeast extract, 20 g/l peptone, 20 g/l glycerol, 20 ml/l ethanol) (SHERMAN *et al.* 1986). The molasses media are based on 6.7 g/l Yeast Nitrogen Base without amino acids (Difco) supplemented with pure molasses (20–400 g/l). Molasses is composed of ~50% sucrose. Two different batches of molasses were used. One was 2 years old (batch 1); the other was 5 mo old (batch 2) and was obtained from another sugar refinery. The concentration of molasses (%) is given in g of molasses per 100 ml of final medium. The molasses media were supplemented with 20 mg/l of tryptophan for the selection of molasses resistant transformants from FL100 (*trp*$^-$, *ura*$^-$).

Escherichia coli strain DH5α was used for routine cloning.

The genotypes of the laboratory yeast strains employed are listed in Table 1. The strains numbered from 1 to 21 are used in industrial ethanol production on molasses or produced on molasses. Their characteristics are listed in Table 2. They all belong to the species *S. cerevisisae.*

The *rtm1-Δ1::URA3* mutation constructed *in vitro* was used to replace the wild-type locus by a one step gene replacement technique (ROTHSTEIN 1983). The 2.8-kb *Sph*I-*Kpn*I fragment derived from a genomic clone (plasmid p1, Figure 1) carrying the entire sequence of *RTM1* was cloned after blunting in the pBluescript SK+ plasmid digested by *Eco*RV. The 0.1-kb *Sal*I fragment located within the coding region of *RTM1* was cut out, and a 1.2-kb *Sal*I fragment containing the *URA3* gene was inserted, yielding the plasmid p1D. *Cla*I and *Bam*HI digestion of this plasmid gives a 2-kb fragment containing the *rtm1-Δ1::URA3* allele (see Figure 1). This fragment was used to replace the wild-type allele of *RTM1* by transformation of the *ura3* haploid and homozygote diploid strains of FL100 background and LG16 (X2180 background). The strain obtained was designed FL100-Δ1.

Growth: The ability of strains to grow on molasses medium was determined on solid medium at 30° during 2–4 days by drop test (3 μl of cell suspension to an A_{600} of 0.2). The minimal concentration of molasses that completely inhibited yeast growth is named MIC (minimal inhibitory concentration).

The growth inhibition by different drugs was performed in the same manner on solid or liquid YNB, YPD or YPGE media.

Cloning of *RTM1* gene: *RTM1* was cloned from a library of 5- to 8-kb *Sau*3A fragments of FL100 genomic DNA inserted into the *Bam*HI site of the pFL44L (BONNEAUD *et al.* 1991), a multicopy based 2μm/*URA3* shuttle vector. The library was kindly provided by F. LACROUTE and F. KARST (unpublished data). FL100 (*ura*$^-$, *trp*$^-$) strain has a MIC of 10% molasses. Putative *RTM1*-containing clones were identified by screening URA transformants (10^8) of FL100 (*ura*$^-$, *trp*$^-$) on 40% molasses. This occurred at a frequency of 1 per 100,000 cells. Proof that the cloned DNA carries a molasses resistance function was obtained by the loss of the phenotype when the strain was

TABLE 2

List of distiller's yeast strains used in French industrial plan for ethanol production

Strain	Origin	Industrial plan
1	UNGDA (Paris)	Distillery no. 1
5	UNGDA (Paris)	Distillery no. 2
6	UNGDA (Paris)	Distillery no. 3
7	UNGDA (Paris)	Distillery no. 4
8	ATCC 2345	
10	ATCC 4126	
11	ATCC 9763	
13		Distillery no. 5
17	INSA (Toulouse)	
18	Yeast producer	
19	Yeast producer	
20	Yeast producer	
21	Yeast producer	

UNGDA, Union Nationale des Groupement de Distillateurs d'Alcool (National Union of Alcohol Distillators); INSA, Institut National des Sciences Appliquées.

cured from the plasmid p1. To subclone the gene-containing fragment, the original genomic clone obtained (p1) was digested with several enzymes (*Sph*I, *Kpn*I, *Bam*HI) and the different generated fragments were cloned individually into adequate sites in the pFL44L. Subclones containing *RTM1* were identified by their ability to confer resistance to 40% molasses medium to the FL100 (*ura*$^-$, *trp*$^-$) strain (Figure 1). The acquisition of the resistant phenotype of the molasses-sensitive strains LG16–1A, LG16–5A and a new clone of FL100 (*ura*$^-$, *trp*$^-$) transformed by p1K was also obtained. The industrial wild-type strain **10** (ATCC 4126) was also transformed by the plasmid p1K-14DM. This plasmid corresponds to p1K where a dominant marker [a mutant allele of the C14 α lanosterol demethylase: $P450_{14DM}$ FLZ^R marker (DOIGNON *et al.* 1993)] is inserted. The transformed strain also shows the resistance phenotype.

Sequencing: DNA sequencing was performed by the chain-termination method (SANGER 1977). For sequencing of the *RTM1* gene, the 2.8-kb *Sph*I-*Kpn*I fragment of the p1K plasmid (see Figure 1) was subcloned into the vector pBluescript SK$^+$ yielding the plasmid ps1K, and several deletions were generated using exonuclease III strategy (ExoIII/Mung Bean nuclease, Stratagene). The whole fragment was sequenced in the two orientations using the Sequenase kit (Pharmacia LKB Biotechnology) under conditions recommended by the manufacturer.

CHEF gel electrophoresis: Growth and preparation of cells for chromosomal DNA analysis are adapted to the protocol described by CARLE and OLSON 1985. A CHEF-2015 PULSATOR TM system-apparatus (LKB) was used to separate the yeast chromosomes. Electrophoresis was carried out at 200 V and 10° for 15 hr with a switching time of 70 sec and then for 12 hr with a switching time of 120 sec. *S. cerevisiae* X2180–1B was used as reference.

Southern blot analysis: After soaking the agarose gels containing the separated chromosomal DNAs or digested fragments of genomic DNA in 0.25 M HCl for 5 min, the DNA was neutralized and transferred to nitrocellulose filters according to the procedure recommended by the manufacturer (Amersham). Hybridization was performed in 5× SSC containing 50% formamide, 5× Dehnhard, 0.5% SDS and 0.2 mg/ml salmon sperm DNA at 45° overnight. The filters were then washed twice with 2× SCC containing 0.1% SDS and with 0.2× SCC containing 0.1% SDS at 65° for 15 min. The probes were labeled with a ^{32}P-dCTP using the random priming kit (Boeringer Mannheim), and detection of hybridization was done by autoradiography. The *RTM1* gene probe was the 1-kb *Hin*dIII-*Bam*HI fragment isolated from the ps1K, the *RTM1* 3′ end region probe was the 1-kb *Bam*HI-*Sst*I fragment isolated from the same plasmid. The *SUC2* gene probe was a 0.8-kb *Hin*dIII-*Bam*HI fragment of the coding region, isolated from the plasmid pRB58 (CARLSON and BOTSTEIN 1982). The *URA3* gene probe was the 1.1-kb *Bgl*II fragment isolated from the plasmid pFL44L.

RESULTS

Cloning of the *RTM1* gene: Plasmids containing the *RTM1* gene have been identified by screening on molasses medium. The strain FL100 (*ura*$^-$, *trp*$^-$) transformed with a yeast (FL100) genomic library of 5- to 8-kb fragments into an 2 μm/*URA3*-multicopy based vector was plated on molasses 40% medium. This concentration of molasses inhibits the growth of FL100 (*ura*$^-$, *trp*$^-$) completely. After 4 days, some transformants were recovered that were resistant to this toxic medium. Plasmid DNA from seven independent transformants was recovered in *E. coli*. Restriction fragment analysis indicated that these seven plasmids contained identical inserts. All of them contained a genomic insert of ~4.5 kb. One of the plasmids (p1) was used for subsequent analysis.

Location of *RTM1* was further defined by different deletions of the plasmid p1. We have subcloned several fragments from the 4.5-kb insert back to pFL44L by using standard techniques to determine the minimal DNA fragment conferring the resistant phenotype. Thus, the plasmids obtained were checked for their ability to confer resistance to 40% molasses medium. The p1K plasmid contained the smallest insert conferring resistance, localizing the *RTM1* gene within a 2.8-kb *Sph*I-*Kpn*I fragment (Figure 1).

Sequence of the *RTM1* gene: The 2.8-kb fragment conferring resistance to molasses was subcloned in pBluescript SK$^+$. Exonuclease III-deleted fragments were sequenced using the Sanger dideoxy method (SANGER *et al.* 1977). Computer analysis of that sequence revealed an open reading frame (ORF) of 927 bp. Figure 2 represents the nucleotide sequence of the *RTM1* gene. This ORF encodes a predicted protein containing 309 amino acid residues with a calculated molecular mass of 34 kD. The primary sequence predicted for the *RTM1* gene product was compared with both GenBank and EMBL data bases using the FASTA and BLASTP algorithms. The search of protein data base failed to detect any significant homologies. Nevertheless, the Rtm1 protein shares a low degree of similarity with putative transmembrane regions of other proteins (30% identical, 50–60% similar over short regions of 20–30 residues). Following analyses of the sequence of Rtm1p (algorithm of KYTE and DOOLITLE 1982; EISENBERG *et al.* 1984), we have found seven predicted high hydrophobic domains that should be potential transmembrane-spanning segments (see Figure 2).

Deletion of the *RTM1* is not lethal: To establish the role of the *RTM1* gene, we have constructed a deletion *rtm1-Δ1::URA3 in vitro,* eliminating a short sequence in the ORF of *RTM1*. This deletion was marked by the *URA3* gene. Construction of the mutant allele of *RTM1* is described in detail in MATERIALS AND METHODS. We tried to introduce the *rtm1-Δ1::URA3* construct by a one-step gene replacement technique (ROTHSTEIN 1983) into two genetic backgrounds. One is derived from FL100 [diploid strain FL100 (*ura3/ura3, trp1/trp1*) and haploid strain FL100 (*ura3, trp1*)]; the other is derived from X2180 background [the diploid strain LG16–2n (*ura3/ura3*) and the haploid strain LG16–5A (*ura3*)]. It was possible to directly produce haploid FL100 URA strains but not haploid or diploid LG16 URA strains. Colonies containing *rtm1-Δ1::URA3* disruption had normal growth rates. Southern hybridization analysis of genomic DNA from the parents and the transformed strains confirmed the disruption of *RTM1*

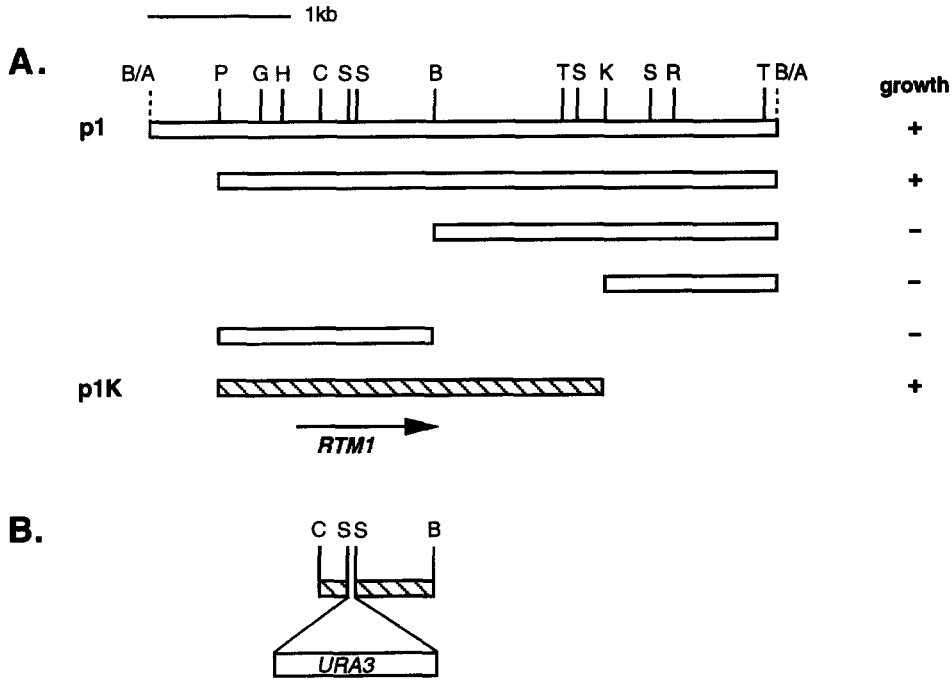

FIGURE 1.—Functional analysis of the subclones from the 4.5-kb genomic fragment of the p1 plasmid containing the *RTM1* gene. (A) Restriction map of the original p1 plasmid. Bars below the map indicate different fragments subcloned in the pFL44L and tested for their ability to confer resistance to 40% molasses medium indicated as growth. The hatched 2.8-kb insert in p1K was the smallest clone defined. (B) Structure of the 2-kb fragment containing the *rtm1-Δ1::URA3* disruption allele. The arrow indicates direction and length of the ORF. A, *Sau*3A; B, *Bam*HI; P, *Sph*I; G, *Bgl*II; H, *Hin*dIII; C, *Cla*I; S, *Sal*I; T, *Sst*I; K, *Kpn*I; R, *Eco*RI.

at the chromosomal locus of FL100 (data not shown), but no *RTM1* hybridized sequences were found in LG16 or X2180, doubtless explaining the failure to produce URA strains by gene replacement procedure. The *RTM1* gene product is therefore not required for cell viability under standard growth conditions.

The *rtm1-Δ1::URA3* disruption strain (FL100-Δ1) was examined for molasses growth phenotype. The deletion of *RTM1* gene in FL100m increased slightly the sensitivity to molasses (shifting the MIC from 10% to 6%).

RTM1 gene does not have a general drug resistance function: We examined four strains for resistance phenotypes that could provide information toward understanding the *RTM1* gene product function. The strains examined were LG16–5A (no *RTM1* gene) and LG16–5A transformed by p1K (Figure 1) (overexpressing *RTM1* gene). We also examined the strains *rtm1-Δ1::URA3*- disrupted FL100 and FL100. Different drugs were tested: substances that could be concentrated in molasses during the sugar extraction process (organic acids, heavy metals), substances that could be added in that process (fungicides, antifoam) and several other toxic substances (Table 3). The *RTM1* gene or the *RTM1* gene on multicopy plasmid does not confer specific resistance to any of the different drug families tested. Thus, its function is not analogous to a general detoxification system. Moreover, we have shown that the aging of molasses leads to an increase in the toxic element concentration (Table 4). The toxicity of molasses is then probably due to one specific inhibiting compound found in several batches of old molasses that is absent or present only at low levels in new molasses.

Genetic diversity of *RTM* genes in yeast strains: As overexpression of *RTM1* allows the growth on molasses and as distiller's yeast used on molasses media show different levels of molasses resistance, we decided to analyze the *RTM1* gene in the genome of those strains. The strains tested are the laboratory strains X2180–1B and FL100 and 13 different yeasts used in industrial ethanol production or produced on molasses (like baker's and distiller's yeasts) (listed in Table 2). Chromosomal DNA from those strains was separated by pulsed field gel electrophoresis, and the chromosomes carrying *RTM* sequence were identified by Southern analysis. Figure 3 shows the hybridization pattern when probed with the sequence derived from the coding region of the *RTM1* gene. Analysis of the karyotype (data not shown) of the 13 industrial yeasts and FL100 shows that their profile is close to the standard strain X2180–1B, but all those industrial strains tested nevertheless show polymorphisms in genomic structure. More striking, all the strains show a different hybridization profile. *RTM* genes are mapped on different chromosomes (chromosomes *IV, VII/(XV), XIII/(XVI), II, VIII/(V)* and two unidentified chromosomes). Particular chromosomes were identified based on their size by comparison to strain X2180 (*II, IV*). Unfortunately, chromosomes *VII* and *XV, XIII* and *XVI, VIII* and *V* usually run as doublets. Therefore we were unable to identify precisely which chromosome hybridizes in those doublets. Nevertheless, different numbers of chromosomes carrying *RTM1* homologous sequences are shown in each strain, except for X2180 where the absence of *RTM1* gene is also confirmed. The different hybridization intensities suggest that a different number of homologous chromosomes carry *RTM* gene. Thus *RTM1* is a member of a new family of genes repeated in the genome as are the *SUC, MAL* or *MEL* families.

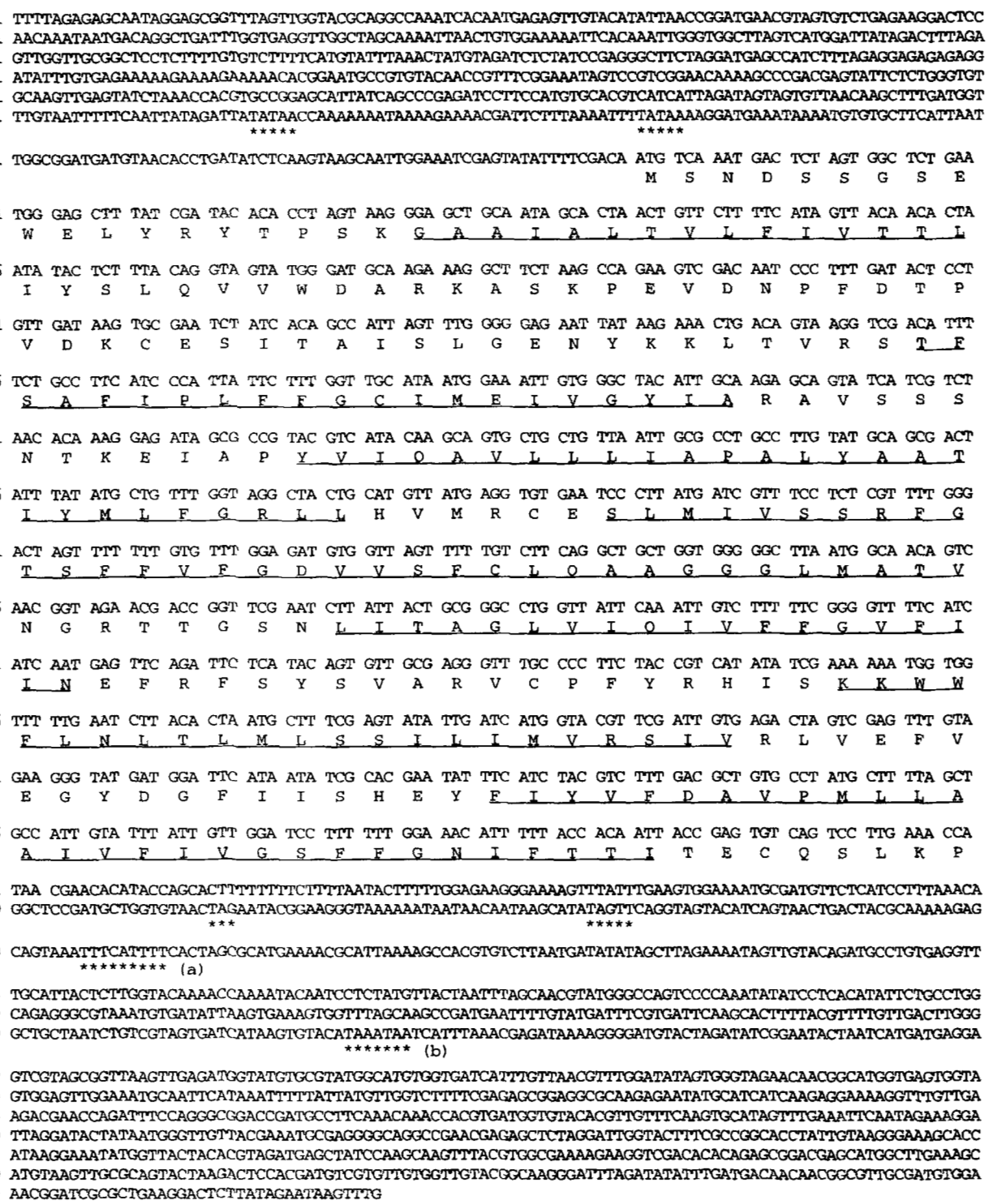

FIGURE 2.—Nucleotide sequence of the *RTM1* gene. The deduced amino acid sequence is indicated. The putative TATA elements and transcription termination signals of (a) ZARET and SHERMAN (1982) and (b) BENNETZEN and HALL (1982) are marked by *. In the amino acid sequence, the seven predicted hydrophobic domains that would be potential membrane spanning segments are underlined. The GenBank accession number is U02618.

Moreover, the minimal copy number of *RTM* genes (estimated from the different location) in yeast is roughly correlated to the molasses resistance (Table 5). Strains carrying at least two copies of *RTM* gene present the higher molasses resistance except strain **5.** This observation is in agreement with the isolation procedure (overexpression) of *RTM1* gene. Moreover, testing the expression of *RTM1* gene by Northern blot, we observed that strains exhibiting the molasses resistance phenotype associated with high copy number of *RTM* sequence show high steady-state levels of the *RTM* transcript when compared to the molasses-sensitive FL100 strain.

TABLE 3

List of the inhibitory compounds tested

Organic acids: acetic, malic, lactic, propionic, benzoic acids
Antifoam: tryptol, Erol XDM1
Heavy metal salts: Cu, Zn, Mn, Mo, Co, Ba, Li
Fungicides: benomyl, nystatin, fenpropimorph, ketokonazol
Others: sinefungin, cycloheximid, tetracyclin, neomycin, actinomycin D

TABLE 4

Measure of the toxicity of different batches of molasses

Batch of molasses	Molasses (%)	Growth of the strains: LG16-5A ($MIC_{batch\ 1}$: 16%)	Growth of the strains: LG16-5A/p1K
1	16	−	+++
	30	−	++
2	30	+	++
	50	+/−	+
2 (after 2 mo.)	16	−	+++
	30	−	++

Yeast growth was performed on solid media as described in MATERIALS AND METHODS. − and + represent in increasing order the growth ability of yeast on molasses media. LG16-5A is used as reference sensitive strain. LG16-5A transformed with the multicopy yeast vector containing *RTM1* gene (p1K) is used as specific resistant yeast to the molasses toxicity.

RTM genes are located in subtelomeric regions: In an attempt to analyze the location of the different copies of *RTM* gene and to be able to understand this gene amplification, we first analyzed the *RTM* loci by hybridization experiments. The genomic DNA from five strains containing different estimated copies (minimal) of *RTM* gene, based on their chromosomal analysis, was digested with different restriction enzymes: strain FL100 (one minimal copy), strain **1** (two minimal copies), strain **5** (five minimal copies), strain **7** (four minimal copies) and strain **13** (seven minimal copies). Using the *RTM1* probe (*Hind*III-*Bam*HI fragment), the genomic *Bam*HI-*Bgl*II digest always generated a single high homology hybridizing fragment of the 1.3-kb expected size (data not shown). Thus, the coding and immediate 5′ region of the different copies is conserved for all the five strains. The hybridization of the *Bam*HI genomic DNA digest by the same *RTM1* probe shows a single fragment of 5.8 kb (Figure 4). The *Bam*HI site in the 5′ side of the different copies of the *RTM* gene is thus conserved in all the strains. The same genomic DNA digestion hybridized with the 3′ side probe of the *RTM1* gene (the *Bam*HI-*Sst*I fragment) gives for FL100, an 8-kb fragment. This result confirms that only one *RTM* locus is present in the strain FL100. For all the other strains a 6.2-kb fragment is observed, and depending on the strains, one, two or perhaps three additional fragments are shown per strain that have respectively 2.5, 1.9 and 2.1 kb. We could therefore define four or perhaps five restriction maps (loci) with these different strains. As the 5′ side and the coding region comprised between the *Bgl*II and *Bam*HI sites are conserved, the variations observed reflect a polymorphism within the 3′ side of the *RTM* gene. To construct more detailed maps, *Hin*dIII and, independently, *Bgl*II genomic DNA digestions were hybridized with the two probes. The results confirm the polymorphism of the 3′ side (see Figure 4). Four distinct loci are described. The fifth hypothetical locus is not presented because it was not possible to locate in this locus the doublet *Hin*dIII *Bgl*II in comparison with the *Bam*HI site in the 3′ side of *RTM*.

In the strain FL100, there is only one locus (locus no. 1). In the other strains tested, a common locus is present (locus no. 2), which is associated with one to three different additional other loci. Moreover, the different intensities of the hydridized bands suggest that the different loci exist in different number. A common chromosome carrying *RTM* shared by the different strains is the chromosome *VII/XV*. It could possibly carry the common locus no. 2. Nevertheless, the number of different loci defined by restriction mapping is not correlated with the number of different chromosomes carrying the *RTM* gene. Then, identical copies of *RTM* could be located on different chromosomes. That homologous chromosomes could share different copies of *RTM* is not excluded by our results.

To determine the identity of the 3′ and 5′ flanking regions, the nucleotide sequence of the 2.8-kb fragment was compared with the GenBank data bases. Surprisingly, we found high homology (80–95% identity) on the 3′ side of the *RTM1* gene (locus isolated from the FL100 genome) with Y′ subtelomeric sequence (see Figure 5). The homology is found with centromere proximal portions of the Y′ subtelomeric sequence described by LOUIS and HABER (1992). The *RTM1* gene is therefore probably located close to the telomere in the following order: centromere . . .-*RTM1*-Y′ sequence-telomere. However, several differences are observed in comparison with the general chromosome end structure. No homology occurred between the nucleotides 1 to 652 and 703 to 1440 of the Y′ sequence. Only a short X subtelomeric sequence portion (of ~50 nucleotides) is found between *RTM1* and the Y′ sequence, the remainder of the X sequence is missing.

As *RTM1* is probably located near a telomere and is a member of a new family of repeated gene, we decided to compare this family with the *SUC* gene family that shares the same characteristics. All the *SUC* genes have highly homologous sequences, are dispersed on different chromosomes (*SUC1, 2, 3, 4, 5* and *7* have been identified respectively on chromosomes *VII, IX, II, XIII, IV* and *VIII*) and are located between the X and the Y′ subtelomeric sequences except *SUC2* (CARLSON *et al.* 1985). The filter used for chromosomal location of

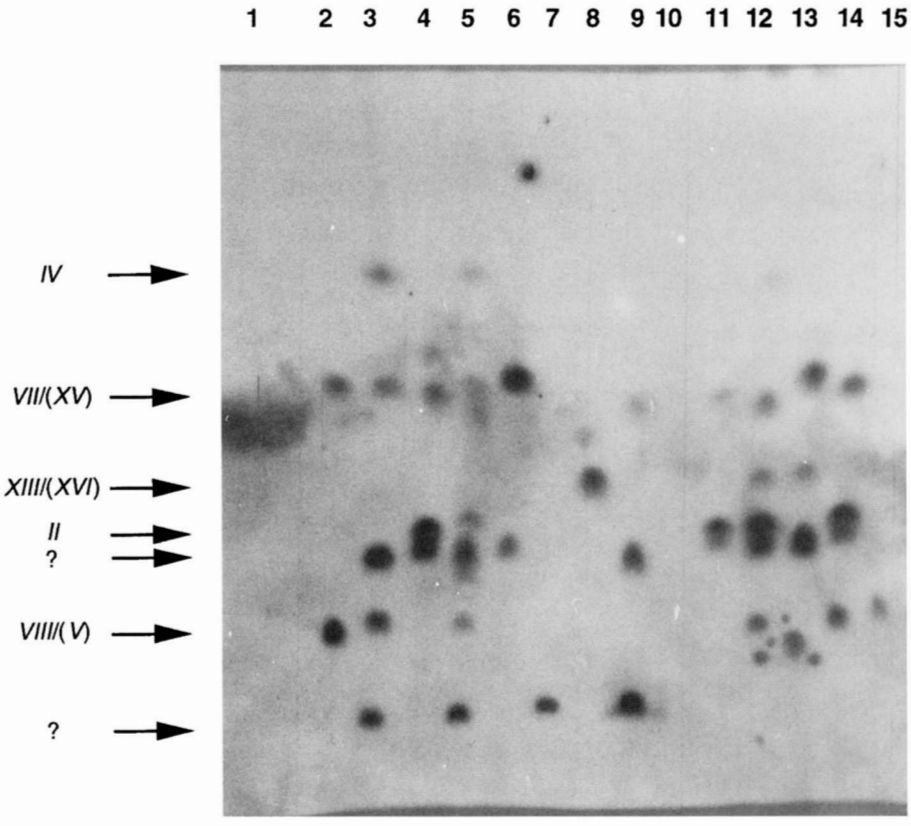

FIGURE 3.—Diversity in copy number and location of *RTM* gene on chromosomal DNAs from different *S. cerevisiae* strains. The figure shows the results of Southern hybridization analysis of the chromosomal DNAs probed with the *RTM1* coding region gene probe. Strains: distiller's yeast strains **1, 5, 7, 13, 6, 8, 10, 11, 17, 18, 19, 20** and **21** (lanes 2–14, respectively), X2180–1B (lane 1) and FL100 (lane 15). Chromosomes estimated to be hybridized are noted.

RTM gene was hybridized with a *SUC2* coding region probe. As the different *SUC* genes share high homology, this probe will hybridize to all of the *SUC* genes. The results (Figure 6) show a high diversity of *SUC* genes (number and location) in the genome of the yeasts used on molasses. All the previously identified *SUC* genes were found in our strains. All of the strains contain a *SUC* sequence on the chromosome *IX* that has been shown to carry the *SUC2* gene. These results are according to the observations of CARLSON and BOTSTEIN (1983). The laboratory strain X2180–1B carries only the *SUC2* gene, and, in addition, the strain FL100 shows the *SUC7* gene on chromosome *VIII*, consistent with the analyses of CARLSON and BOTSTEIN (1983) and CARLSON *et al.* (1981). The different industrial yeasts share the common *SUC2* gene and from one to five different telomeric *SUC* genes (*SUC1, 3, 4, 5* or *7*). Moreover, two unidentified locations are found.

TABLE 5

Relation between natural *RTM* copy number and molasses-resistant phenotype

Strain	Minimal copy number	$CMI_{batch\ 1}$ (% molasses)
X	0	16
17	0	20
FL	1	20
10	1	8
8	1	30
11	3	20
1	2	>40
6	2	>40
18	3	≥40
20	4	≥40
21	4	≥40
7	4	>40
13	7	40
19	5	>40
5	5	20

Estimation of minimal *RTM* copies is based on the number of chromosomal bands (see Figure 3) hybridizing to *RTM1* probe. Molasses-resistant phenotype is expressed in MIC (%). Strains: X, (X2180-1B); FL, FL100; numbered strains, industrial distiller's strains.

The comparison between *RTM* and *SUC* hybridization chromosome profiles show a remarkable result. All the chromosomes hybridized with the *RTM1* probe are also hybridized with the *SUC2* probe. These chromosomes are therefore the chromosomes carrying *SUC* telomeric gene (*VIII, II, XIII, VII* and *IV*) and the two unidentified chromosomes. The chromosome *IX* known to carry the nontelomeric *SUC2* gene is the only chromosome not hybridized with the *RTM1* probe in all the strains tested. Therefore the presence of the *RTM* gene is always associated with *SUC* telomeric genes. Moreover, the hybridization intensities are the same with *RTM1* and *SUC2* probes. This observation suggests that the copy number of *RTM1* and *SUC* genes are the same on each group of homologous chromosomes concerned.

As *RTM* repeated sequences and *SUC* telomeric genes

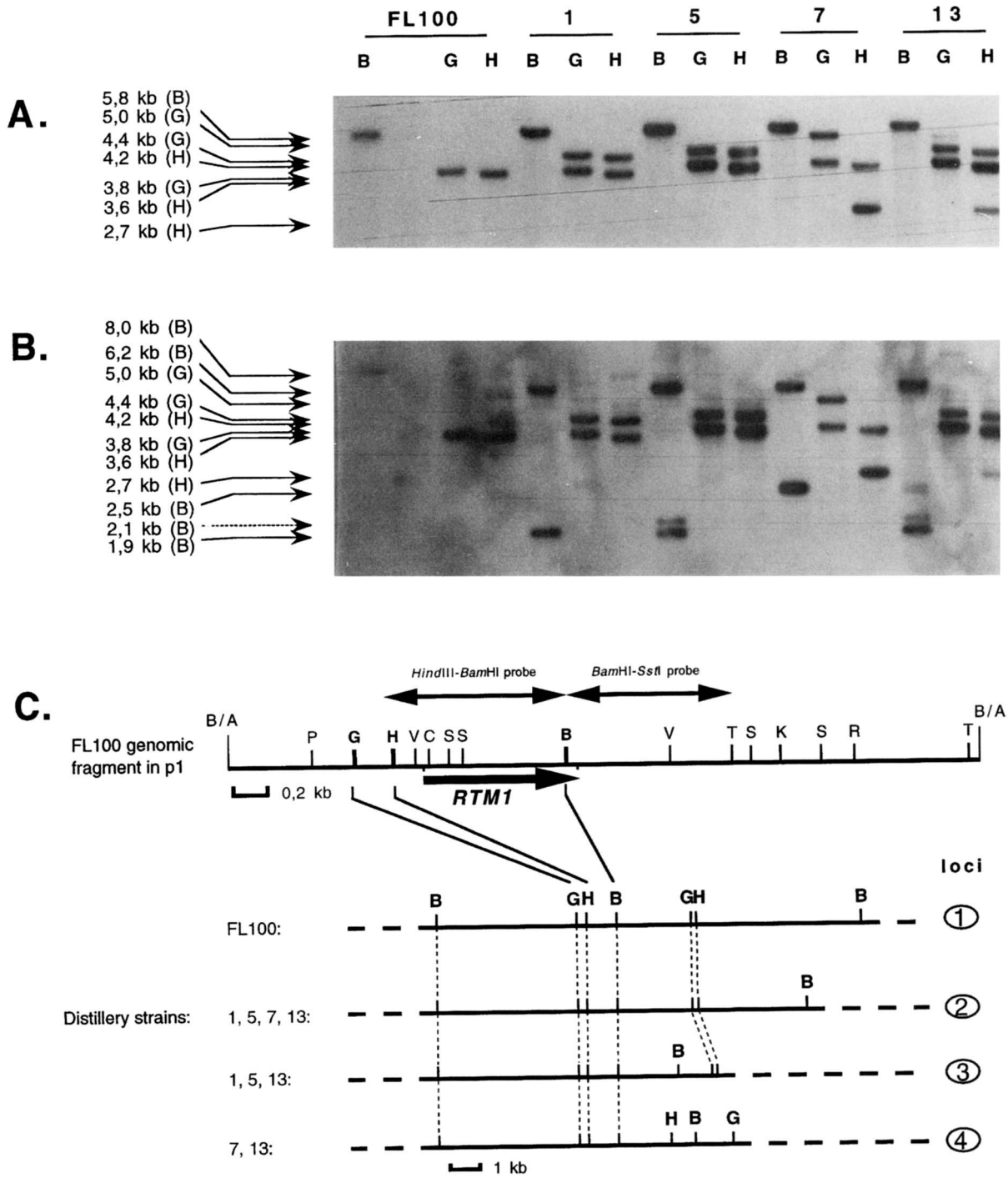

FIGURE 4.—Southern analysis of the different *RTM* copies in different yeast strains. The genomic DNA from the FL100 strain and the distiller's strains **1, 5, 7** and **13** were digested with *Bam*HI (lane B), *Bgl*II (lane G) or *Hin*dIII (lane H) and hybridized with the *Hin*dIII-*Bam*HI fragment (*RTM1* gene) (A) and the *Bam*HI-*Sst*I fragment (3' side of *RTM1* gene) (B). (C) Schematic representation of the different loci present in FL100 and distiller's strains deduced from the Southern analysis. Restriction sites: A, *Sau*3A; B, *Bam*HI; P, *Sph*I; G, *Bgl*II; H, *Hin*dIII; C, *Cla*I; V, *Eco*RV; S, *Sal*I; T, *Sst*I; K, *Kpn*I; R, *Eco*RI.

seem to be carried by the same chromosomes and as these genes are located near the Y′ subtelomeric sequences, we decided to compare the *RTM1* restriction map and those of the *SUC* genes established by CARLSON *et al.* (1985). The results (Figure 7) show that the restriction sites defined on *RTM1* gene correspond to the conserved restriction sites located between the different *SUC* telomeric genes (and in particular the *SUC7*) and the Y′ sequence. The *Bam*HI site located on the 5′ side of the *RTM1* gene and conserved in the different loci we have defined (Figure 4) corresponds to the *Bam*HI site in the coding region of the *SUC* sequence. The *Sal*I and *Eco*RI sites identified in the 3′ side of *RTM1* in the Y′ homologue sequence are conserved sites present in

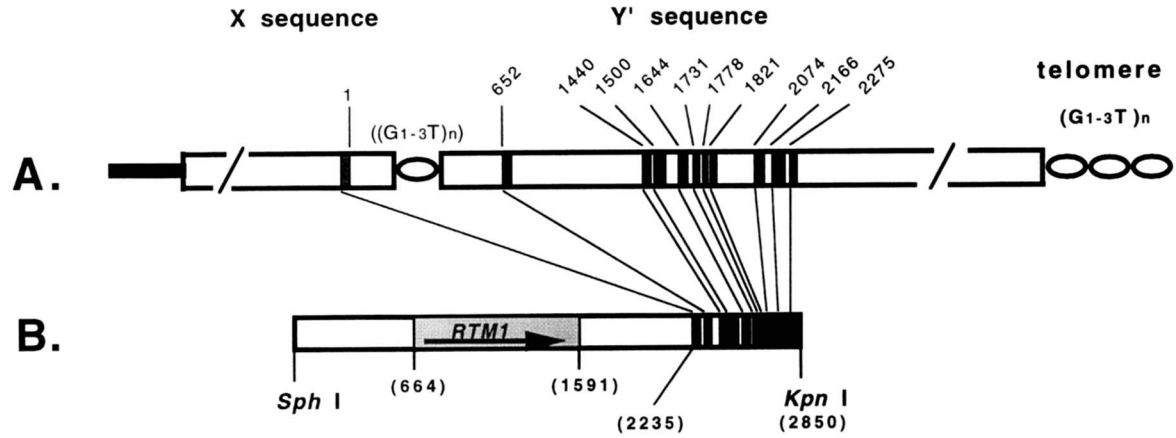

FIGURE 5.—Comparison of the nucleotide sequence of the 3′ side of the *RTM1* gene and subtelomeric sequences. Schematic representation of *RTM1* 3′ side homology (80–95%) with subtelomeric portions of Y′ (LOUIS and HABER 1992) and X (BUTTON and ASTELL 1986) sequences. (A) Chromosome end in *S. cerevisiae*. Oval symbols represent functional telomere composed of (G1–3T)n repeats. Adjacent to this sequence are up to four tandem copies of the Y′ element. This element can be missing. These are then preceded by the X element. The telomere sequences are sometimes found between X and Y′. The sequence is numbered like this in LOUIS and HABER (1992). (B) The 2.8-kb *Sph*I-*Kpn*I fragment (sequence see Figure 2).

the different proximal Y′ sequence analyzed near the *SUC* telomeric genes. Therefore the *RTM1* gene is physically associated with *SUC* telomeric genes (*SUC7* in the strain FL100) between the X and the Y′ subtelomeric sequences. As the *RTM* gene and the 5′ side of *RTM* are completely conserved in the different loci defined in the different strains tested (FL100, 1, 5, 7 and 13), all the dispersed *RTM* sequences have the same location related to *SUC* genes. The 3′ side *RTM* polymorphism probably corresponds to the Y′ sequence variation.

DISCUSSION

We have identified a new gene called *RTM1* dispersed throughout the yeast genome. The different *RTM* sequences are highly homologous and are always physi-

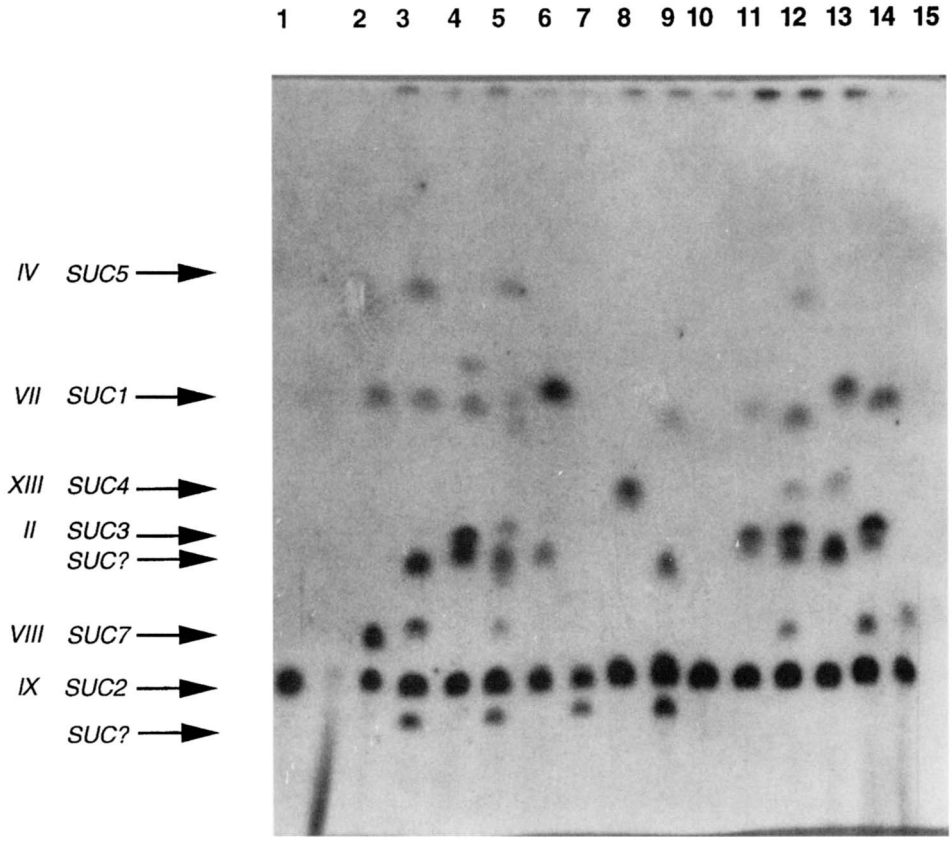

FIGURE 6.—Southern hybridization analysis of chromosomal DNAs from different *S. cerevisiae* strains. The figure shows the results of Southern hybridization analysis of the chromosomal DNAs probed with a *SUC2*-coding region gene probe. The membrane used was the same as the one used for *RTM* Southern analysis (Figure 3) after total dehybridization. Strains: distiller's yeast strains **1, 5, 7, 13, 6, 8, 10, 11, 17, 18, 19, 20** and **21** (lanes 2–14, respectively), X2180–1B (lane 1) and FL100 (lane 15).

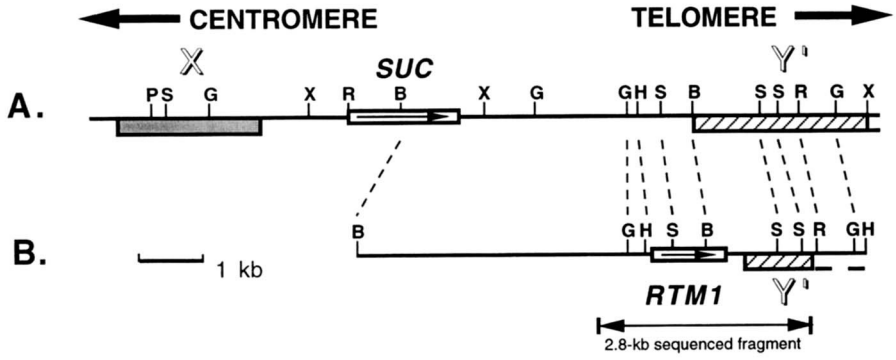

FIGURE 7.—Comparison of the structure of the *SUC* telomeric and *RTM1* loci. (A) Conserved structure of the different *SUC* telomeric loci (*SUC1, 3, 4, 5, 7*). The restriction sites represented are the conserved or majority sites between the different *SUC* loci (CARLSON *et al.* 1985). (B) Restriction map of *RTM1* loci in FL100 strain. The *SUC, RTM1,* Y′ and X sequences are represented as boxes. Arrows indicate *SUC* and *RTM1* structure genes and are pointed in the direction of transcription. The 2.8-kb sequenced fragment is shown. Restriction sites: P, *Sph*I; S, *Sal*I; G, *Bgl*II; X, *Xba*I; R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

cally associated with *SUC* telomeric genes; each associated *SUC-RTM* sequence is inserted between X and Y′ subtelomeric sequences, *i.e.,* centromere. . .-X sequence-*SUC-RTM*-Y′ sequence-telomere. Transcription of the two genes is in the same centromere-to-telomere direction.

The fact that *RTM* sequences are adjacent to Y′ sequences could explain the 3′ side of *RTM* loci's polymorphism. One hypothesis is that the Y′ variation includes many insertion/deletions as described by LOUIS and HABER (1990b, 1992) and NAUMOV *et al.* (1992). Another hypothesis could be the variation of *SUC RTM* sequence's insertion position at the 5′ side of the Y′ sequence.

The high number and location diversity and the particular common insertion of these associated *SUC-RTM* sequences seem to indicate that the dispersal of *SUC-RTM* sequences to different chromosomes occurred by rearrangements of chromosome termini. CARLSON *et al.* (1985) suggested a model for the evolution of the telomeric members of the *SUC* gene family in regard to the comparaison of the *SUC* loci and the *suc*° loci (loci lacking the *SUC* gene). This model could then be adapted not only to the *SUC* gene but to the whole *SUC-RTM* sequence. The model proposed that the first telomeric *SUC* locus evolved by the insertion of an ~7-kb element containing a *SUC* and a *RTM* gene between X and Y′ subtelomeric sequences. The resulting *SUC-RTM* locus could then move to different chromosomes by recombination or could be transferred by conversion mediated by the homology of the subtelomeric sequences. The evolutionary relationship between the nontelomeric *SUC2* and the other *SUC* genes, suggested by CARLSON *et al.* (1985), is a gene duplication where *SUC2* is speculated to be the ancestral gene, but the lack of *RTM* gene near the *SUC2* gene adds another variable to the model. It could be proposed that the first duplication event of *SUC2* corresponds to the insertion of *SUC2* in an *RTM* original telomeric locus. The two genes were then dispersed together through other telomeres. Nevertheless such an isolated *RTM* locus is never seen in our study. It is noteworthy that these 7-kb *SUC-RTM* sequences are present only at subtelomeric loci in all the strains tested. This sequence is not found in the strain X2180 nor its progenitor S288C (CARLSON *et al.* 1985) where the only *SUC* locus, *SUC2,* is not telomeric.

The lack of the *RTM* sequence in some strains raises the question of the *RTM* origin. The same remark could be asked for Y′ subtelomeric sequences. Y′ elements are not distributed widely among yeasts (JÄGER and PHILIPPSEN 1989; NAUMOV *et al.* 1992). In *S. cerevisiae* these elements are highly conserved, are adjacent to the telomere, and present in one to four tandem copies, but are not found at all chromosome ends (SZOSTAK and BLACKBURN 1982; CHAN and TYE 1983; WALMSLEY *et al.* 1984; LOUIS and HABER 1990a). Several structural features are consistent with a mobile element origin for Y′s (LOUIS and HABER 1992). These characteristics support perhaps a link between *SUC-RTM* and Y′s evolution or dispersion. But even if their dispersion among the genome could have occurred with the same mechanism (by recombination or conversion), they have evolved independently. Indeed, *SUC-RTM* is always found adjacent to the Y′ sequence (this study and CARLSON *et al.* 1985) but the Y′ sequence could be present without the *SUC-RTM* sequence, *e.g.,* in the strain S288C (see LOUIS *et al.* 1994).

In *S. cerevisiae* other repeated gene families have been described [*MAL* (CHARRON *et al.* 1989), *MEL* (NAUMOV *et al.* 1990, 1991)]. A common characteristic can be highlighted between the two telomeric *RTM* and *MEL* families compared to the other gene's family. This is the lack in some strains of any *RTM* or *MEL* sequence, when in all strains tested there is at least a *SUC2* gene (CARLSON and BOTSTEIN 1983) and at least an allele of *MAL1* (NAUMOV *et al.* 1994). This observation may suggest a similar evolution mechanism. Their amplification is therefore independent because all the *MEL* genes seem to be located on left ends of chromosomes (TURAKAINEN *et al.* 1993), when *SUC* telomeric genes mapped are located on right ends of chromosomes (MORTIMER *et al.* 1992). Ends of chromosomes seem then to be a good way for spontaneous gene amplification.

A recent publication concerning the *MEL* gene family describes a new unidentified ORF that like the *RTM* genes is absent or present in different copy number in yeast (TURAKAINEN *et al.* 1994).

The *RTM* gene amplification may have a real functional role. *RTM1* overexpression provides a higher level of resistance on molasses media. It is noteworthy that compared to laboratory strains (0 or 1 *RTM* copy), the industrial strains grown on molasses (biomass production or industrial ethanol production) show the amplification of *RTM* sequences throughout the genome. The high copy numbers of this sequence in distiller's yeasts is correlated with the high transcription rate compared to the FL100 strain (one *RTM* copy), even if *RTM* sequences are located near telomeres described to repress the transcription (GOTTSCHLING *et al.* 1990). Indeed, this amplification can explain the adaptation of the growth of these yeasts on molasses. The *RTM* genes show genetic diversity in this population of yeasts and seem to be a good example of genomic rearrangement playing a role in evolution and environmental adaptation in yeast population used on molasses. This remark includes the physically associated telomeric *SUC* genes encoding the invertase enzyme by the fact that the major carbon source in beet molasses is sucrose. Indeed, changes in yeast genomic structure in relation to the culture conditions were observed by ADAMS *et al.* (1992).

In contrast to distiller's strains, yeasts originally not growing on molasses like the majority of wine strains, show only the *SUC2* gene and no *SUC* telomeric gene (BIDENNE *et al.* 1992). It can be noted that those yeasts ferment grape juice that contains glucose and fructose instead of sucrose for molasses. We did not test the presence of *RTM* in these strains but suspect its absence as *SUC* telomeric genes are absent too.

The Rtm1 protein does not belong to the family of multidrug resistant proteins that are in most cases large proteins with 12 transmembrane domains. It seems that Rtm1p does not provide a general detoxification function but confers a resistance to a particular toxic element present in some molasses. This toxic element appears with time in beet molasses. The Rtm1 protein looks like a member of the seven transmembrane segment protein family (for review see DOHLMAN *et al.* 1991). It could be, for example, a signal-transducing protein like *STE2* or *STE3* (BURKHOLDER and HARTWELL 1985; HAGEN *et al.* 1986) or a specific extrusion pump. Codon usage of the *RTM1* gene is surprisingly low (CAI = 0.093) (SHARP and COWE 1991). This value is closer to those of genes encoding regulation or signal transduction proteins than for genes encoding enzymes or transporters. The identification of the toxic element would give us a tool to study the function of this membrane protein encoded by this new gene family.

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