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Industrial Yeasts Display Tandem Gene Iteration at the *CUP1* Region

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The gene copy number at the *CUP1* locus and the resistance level to external copper was directly correlated in five wild-type commercial *Saccharomyces* strains. An increased copy number of the *CUP1* gene leads to increased accumulation of chelatin mRNA, which codes for a low-molecular-weight, copper-binding protein. The enhanced production of this rapidly inducible protein mediates resistance of the cell to copper. Industrial yeasts exhibit homologies to the amplified copper resistance repeat unit found in laboratory strains. However, the extent of tandem iteration is strain dependent, and the repetitious unit is either 1.7 or 1.5 kilobases in length compared with the 2.0-kilobase unit in laboratory strains. Strain 522 (Montrachet) contains two chromosome VIII segments distinguishable by their numbers of repeat units (2 and 11) and the size of the units (1.5 and 1.7 kilobases). Distillers yeast 513 carries a 1.5-kilobase repeat unit on each homologous chromosome, although they contain nine and five iterations, respectively.

Gene amplification or increase in the copy number of the gene is a common adaptive mechanism exploited by different organisms to increase their resistance to various toxic and antiproliferative agents. A particularly well-studied example of the gene amplification process involves the increased resistance to methotrexate exhibited by cultured mammalian cells. Here, amplification of the dihydrofolate reductase gene (for review, see reference 11) mediates the enhanced resistance to the environmental challenge. Other notable examples include the enhanced resistance to *N*-(phosphonacetyl)-L-aspartate mediated by amplification of the *CAD* gene (17) and the elevated resistance of cultured mouse cells to cadmium mediated by the amplification of the metallothionein gene (1).

Our studies concern the genetic and molecular mechanisms leading to development of resistance to copper ions in yeasts. A genetic locus responsible for copper resistance in various laboratory strains of the yeast *Saccharomyces cerevisiae* was reported nearly 30 years ago (2). Naturally occurring copper-sensitive strains were described to have the *cup1*^{s} allele, and resistant strains carried the corresponding *CUP1*^{r} allele at the same genetic locus. Extensive analysis of unselected tetrads generated from hybrids between *CUP1*^{r} and *cup1*^{s} strains demonstrated that the level of copper resistance comprises a difference under the control of a single Mendelian unit. Assignable to a chromosome VIII site, the locus is situated some 40 to 50 centimorgans from its respective centromere and exhibits a second division segregation frequency equal to 76% (7). The *CUP1*^{r} allele is semidominant, and the *CUP1*^{r}/*cup1*^{s} heterozygote displays an intermediate resistance level relative to the corresponding parental homozygotes.

Recently, we isolated the *CUP1*^{r} gene by transforming an appropriately marked *cup1*^{s} recipient to copper resistance (6) with a collection of hybrid plasmids constructed by the insertion of random yeast genomic DNA fragments into the vector YRp7 (14). The transformants display all of the properties expected of cells containing an autonomously replicating plasmid. Extensive genetic analysis established that integration of the plasmid into the genomic DNA occurred preferentially at the *cup1*^{s} locus on chromosome VIII which is 28 centimorgans distal to the *thr1* marker. Restriction enzyme studies established that the *CUP1*^{r} locus is a tandemly iterated, 2.0-kilobase (kb) sequence containing single *Kpn*I and *Xba*I sites and two *Sau*3A sites. The *CUP1*^{r} allele embraces 10 iterated sequences of the fundamental repeat unit, in contrast to the sensitive allele (*cup1*^{s}), which contains only a single

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unit (6). The complete nucleotide sequence of the cloned repeat unit was determined, and we will report it elsewhere. It contains an open reading frame, coding for a low-molecular-weight, cysteine-rich, copper-binding protein known as copper chelatin (9). Of the 60 amino acids encoded, 10 were cysteine.

A cloned 2.0-kb DNA fragment confers copper resistance. Using this as a hybridization probe, we determined the gene copy number and production of chelatin mRNA in various yeast strains. Originally isolated from nature, these strains are now propagated commercially. Here, we report that some industrial resistant strains contain increased copy numbers of the CUP1 gene relative to sensitive strains. The resistant strains also produce more chelatin mRNA, which is regulated in turn by the level of copper ions in the growth medium. Reported also are the findings that various commercial strains used for industrial wine or whiskey production differ both with respect to the length of the repeat unit as well as the extent to which the same unit is iterated through a gene amplification mechanism.

MATERIALS AND METHODS

Yeast strains. Most of the yeast cultures listed below were generously supplied by H. Phaff and M. Miranda at the Department of Food Technology, University of California, Davis. Strains 505 and 522 were kindly supplied by R. E. Kunkee at the Department of Viticulture and Enology, University of California, Davis. Strain designations and their respective culture numbers are cited in Table 1.

X2180-1A is a standard wild-type copper-resistant strain that carries the *CUP1*^{r} allele. JW251-1A is a laboratory copper-sensitive strain with the allele *cup1*^{s}. Both were *S. cerevisiae* strains.

Media. Yeast culture conditions were previously reported (5, 6). Copper plates were made with synthetic complete medium solidified with 1.5% Phytagar (GIBCO Laboratories) (6). Liquid copper media typically contained lower copper concentration levels than plates since the ion is less toxic in the presence of agar.

Analysis of copper resistance. Copper resistance levels were determined by assaying for confluent growth after 2 days on plates with increasing concentrations of copper.

RNA analysis. Harvested cells were disrupted in a small volume of 5% polyvinylpyrrolidone-360–20% glycerol–1 M sorbitol–0.5% sodium dodecyl sulfate in a prechilled French pressure cell at 15,000 lb/in^2 of pressure. Broken cell preparations were stored at −70°C until RNA extraction was performed. The broken cells were extracted in 5 M guanidinium isothiocyanate–50 mM Tris-hydrochloride–10 mM EDTA (pH 7.5), and RNA was precipitated overnight at 4°C by addition of LiCl to final concentration of 3.25 M. RNA pellets were washed in 3 M LiCl, suspended in 10 mM Tris-hydrochloride–1 mM EDTA–0.1% sodium dodecyl sulfate, and extracted once with a phenol–chloroform–isoamyl alcohol (24:24:1) mixture.

RNA was finally collected by ethanol precipitation and dissolved in a small volume of water. RNA concentration was determined by optical density measurements, and 10-μg samples were analyzed after glyoxalation on 1.5% agarose gels as described by Thomas (16).

Nitrocellulose filters were hybridized to ^{32}P-labeled DNA probes that were prepared by nick translation (10). Conditions for hybridization were as described by Wahl et al. (18). ^{32}P-labeled DNA probes were prepared from the 2.0-kb *Xba-Xba* DNA fragment (one complete repeat unit) and from the 0.7-kb *Xba-Kpn* DNA fragment (the repeated segment containing the entire CUP1 gene; see Fig. 1) (6). The former

TABLE 1. Gene copy number and copper resistance levels of various yeast strains[a]

Strain		Approx gene copy no. per diploid genome	Minimal copper concn inhibitory to growth (mM)
513	*S. cerevisiae* Distillers	9 + 5	0.8
505	*Saccharomyces bayanus* California Champagne	4 + 4	0.4
519	*Saccharomyces fermentati* Sherry Flor	1 + 1	0.1
522	*S. cerevisiae* Montrachet	11 + 2	0.8
66-1051	*Saccharomyces oleaginosus*	9 + 9	1.4
68-917B	*Saccharomyces kluyveri*	—	1.8
JW251-1A	*S. cerevisiae* laboratory strain	1 + 1	0.15
X2180-1A	*S. cerevisiae* laboratory strain	10 + 10	2.0
YNN53	*Saccharomycodes ludwigii*	—	2.0

[a] Ploidy levels are unknown. Thus, strains displaying two *Eco*RI bands (Fig. 2) are assumed to be at least diploid. Gene copy numbers for haploid strains or strains exhibiting a single band were doubled to yield comparable values. Haploid and corresponding homozygous diploid laboratory strains display the same copper resistance levels. The calculations that establish copy numbers are given in the results section. Genetic background significantly modifies the copper resistance level. Only isogenic strains exhibit a strict correlation between resistance and gene copy number. Thus, comparisons between wild-type strains are, at best, approximate.

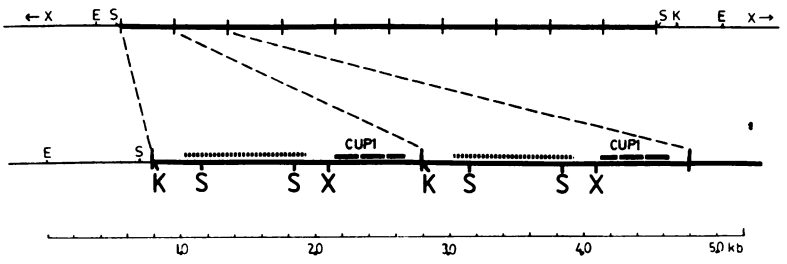

FIG. 1. Restriction map of a *CUP1*^{r} X2180-1A. The thick solid line segment represents the CUP1 locus as 10 tandemly iterated 2.0-kb units, with each unit marked by vertical lines. The thin line segments represent the external flanking DNA sequences. Restriction sites within the flanking regions are given as smaller letters placed above the thin line. *Xba* sites in the flanking region are 11 and 21 kb from the repeat region. Shown also are two expanded repeat units. Sites within the repeat region are indicated by larger letters below the thick line: X, *Xba*I; K, *Kpn*I; E, *Eco*RI; S, *Sau*3A. *Xba-Xba* intervals are 2.0 kb, and *Kpn-Kpn* intervals are 2.0 kb. Junction fragments are: *Kpn*, 2.7 kb; *Xba-Eco*RI, 2.1 and 3.1 kb. Our unpublished DNA sequence identifies two open reading frames corresponding to separate coding regions. Each region expresses a unique mRNA transcript. The thick dashed line denotes the coding region of the chelatin gene. The dotted line denotes the coding region of an unknown gene. Probes were made from the *Xba-Xba* 2.0-kb and the *Xba-Kpn* 0.7-kb DNA fragments from cloned CUP1 DNA.

fragment was used for all DNA hybridizations, and the latter fragment was used for DNA-RNA hybridizations.

DNA analysis. Total genomic DNA was isolated as described by Struhl et al. (14). Restriction digests were carried out according to manufacturer recommendations (Bethesda Research Laboratories or New England Bio-Labs). DNA was analyzed by agarose gel electrophoresis and blot hybridization as described by Southern (12). Hybridization conditions were as described previously (13).

Notation. The symbols and notation required to convey the major molecular, genetic, and phenotypic aspects of copper resistance in yeasts are somewhat complex. This arises from our finding that even though for resistance and sensitivity to copper the alleles behave and segregate as a simple Mendelian unit, the allelic differences at the DNA level are quantitative rather than qualitative. Resistant strains harbor several tandemly arrayed, repetitive sequences. In contrast, sensitive strains carry only a single unit at the corresponding chromosomal site. Genetically determined resistance assignable to the appropriate chromosome VIII site is designated as *CUP1*^{r}. This implies gene amplification of the coding sequence for copper chelatin to various extents among the laboratory and industrial *Saccharomyces* strains. The symbol *cup1*^{s} denotes a single copy of the basic unit. The symbol CUP1 (not italicized) may refer to single or multiple copies of either the entire repeat unit or the chelatin coding sequence alone.

Resistance to exogenous copper is a graded rather than an "all or none" phenotypic response, and the resistance level attained by any given strain is determined by the gene copy number at the locus, as well as the copper concentration used to induce the chelatin protein production.

RESULTS

To assess the extent of genetic diversity in various industrial *Saccharomyces* strains with respect to copper resistance, we first determined the minimal copper ion concentration inhibitory to the growth of those strains. As indicated in Table 1, the various strains exhibited characteristic levels of copper resistance over a 20-fold range. As a basis for comparison, we also included two laboratory strains that are markedly different in their copper resistance. The copper-sensitive haploid laboratory strain JW251-1A carries only a single copy of the CUP1 gene, whereas the corresponding haploid-resistant strain X2180-1A bears 10 iterated copies of the same sequence (6). To determine whether the observed differences in copper resistance among commercial distillers and wine yeast strains is also mirrored by corresponding differences in copy number of the CUP1 gene, we analyzed genomic DNA by DNA hybridization according to the method of Southern (12).

The size of the CUP1 locus was determined by blot hybridization of DNA cleaved with *Eco*RI, which does not cleave within the basic repeat unit. The DNA hybridization pattern of *Eco*RI-digested DNA from the different strains to a CUP1-specific probe is shown in Fig. 2. Since *Eco*RI cuts only within the flanking DNA, the overall size of the *Eco*RI fragment containing the entire CUP1 region should be directly proportional to the number of tandemly iterated units contained within the locus. In *Eco*RI digests of genomic DNA from JW241-1A, a copper-sensitive laboratory strain containing a single copy of the CUP1 gene, the locus is detected as a 5.2-kb fragment. This represents a single fundamental repeat unit of 2.0 kb and two flanking segments of 0.8 and 2.4 kb (6). In contrast, X2180-1A, a resistant laboratory strain, contains 10 copies of the same CUP1 gene, and the

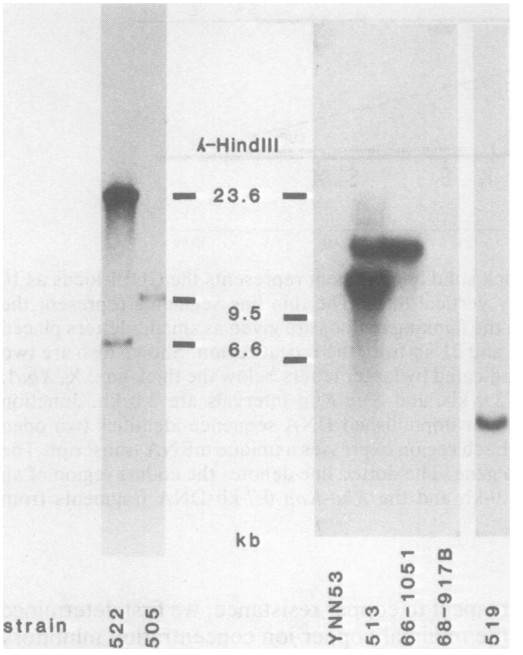

FIG. 2. DNA hybridizations of *Eco*RI-digested genomic DNA (about 20 to 40 μg per lane) from commercial yeast strains. λ-*Hin*dIII marker bands and lengths are given between the two autoradiograms.

segment is present as a 23-kb *Eco*RI fragment. Most naturally occurring wine strains also display *Eco*RI bands hybridizing to the CUP1-specific probe. However, although their molecular lengths are distinctively different, they correlate positively with the copper resistance level of the strain. Strains with higher copper resistance levels have longer *Eco*RI fragments containing the CUP1 genes. Note in Fig. 2 that digests from two strains, YNN53 and 68-917B, do not contain DNA fragments with detectable homology to the CUP1 probe under conditions of high stringency.

In the haploid laboratory strains, JW251-1A and X2180-1A, the CUP1 genes are detected as a single *Eco*RI band, indicating the presence of only a single cluster of tandemly iterated genes. In contrast to the laboratory strains, two among five commercially propagated industrial strains contain more than a single *Eco*RI fragment that hybridizes with the CUP1 probe (Fig. 2).

To determine the size of the individual repeat units in the various strains, we digested genomic DNA with both *Xba*I and *Eco*I. Each iterated unit contains one *Xba*I site (6). Digestion of genomic DNA from the laboratory strains containing tandemly iterated units with *Xba*I and *Eco*I generates a multimolar band at 2.0 kb corresponding to the physical size of the basic repeat unit. In addition, two fainter unimolar bands were identified by the same CUP1 probe. Equivalent unimolar fragments are detected in genomic DNA digests from sensitive strains containing only a single CUP1 gene. Such bands correspond to DNA segments extending from the *Xba*I site within the repeat unit to a neighboring *Eco*RI site in the flanking DNA. Genomic DNA from commercial wine yeast strains was digested with *Xba*I and *Eco*RI, separated by agarose gel electrophoresis, and transferred to nitrocellulose. DNA samples from wine strains 505, 522, 513, and 66-1051 each contained three distinct bands (522 had four bands) that hybridized with the CUP1 probe (Fig. 3). The intense band corresponds to the basic repeat unit of the CUP1 locus, whereas the fainter bands represent the flanking or junction fragments. DNA from the sensitive laboratory strain JW251-1A contains only a single copy of the CUP1 gene. Predictably, it displays only the two junction bands since the chelatin-coding sequence is not

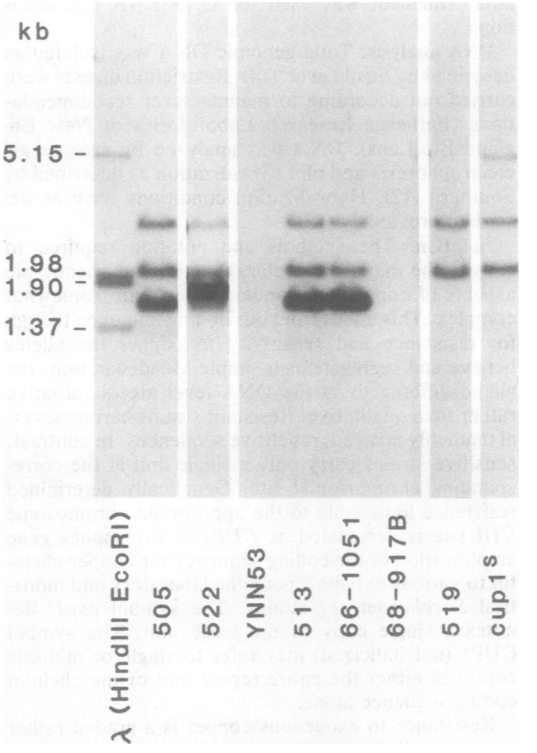

FIG. 3. DNA hybridizations of *Xba-Eco*RI digests (20 to 40 μg of DNA per lane). Strains 505, 522, 513, and 66-1051 show multimolar 1.5-kb bands. Strain 522 also has a multimolar 1.7-kb band. All hybridizable strains have junction bands of 2.1 and 3.1 kb which are identical to the *cup1*^{s} laboratory strain (the 5.2-kb band in *cup1*^{s} indicates a partial digestion). Different exposures with matched intensities of the single copy junction bands are juxtaposed.

amplified. Since strain 519 shows only two bands, it must also possess a single copy of the CUP1 gene. All other strains display three bands that hybridize to the CUP1 probe, indicating they contain multiple repeat units.

We emphasize that, although the lengths of the junction bands are identical in all strains containing hybridizable DNA, the physical length of the basic repeat unit is characteristically different in each strain. Strains 505, 513, and 66-1051 all display repeat units of 1.5 kb. However, Montrachet strain 522 manifests an intense multimolar band migrating at 1.7 kb, as well as a fainter 1.5-kb band. These values are significantly different from the 2.0-kb repeat unit found in the wild-type laboratory strain X2180-1A. We note that the *Eco*RI bands (Fig. 2) in strains 505 and 513 each contain the same 1.5-kb repeat unit. But, in strain 522, which is characterized by a 1.7-kb as well as a 1.5-kb repeat unit, we detected two DNA segments that were amplified to different extents. This result derives from hybridization performed on digested genomic DNA taken from the ascosporal colonies of unselected tetrads. Segregation analyses reveal that 23- and 6.6-kb bands are distributed in a pattern consistent with the assumption that strain 522 is at least diploid. Thus, a given ascosporal culture may contain either single band alone. A spore culture carrying the 23-kb band yields 1.7- and 1.5-kb repeat units upon digestion with *Xba*I, whereas sister spore cultures carrying the 6.6-kb band release only the 1.5-kb repeat unit.

The number of CUP1 genes in each strain was determined as follows. The distances between the left and right *Eco*RI sites and the corresponding ends of the repeat unit are 1.3 and 2.4 kb for strains with the 1.5-kb repeat. The sum of these values, 3.7 kb, is subtracted from the overall length of a hybridizable genomic *Eco*RI band of the strain. When this value is divided by the repeat unit length in kb, we generate the number of repeat units. Similar calculations apply for determining the copy number of the 1.7-kb repeat unit.

As indicated in Table 1, a direct correlation exists between the number of CUP1 genes in a given strain and the level of copper resistance of the strains. The effect of increasing the number of repeat units on chelatin mRNA production was assayed in two widely used wine strains, 505 and 522. Total RNA from cells grown in the absence of copper, or in the presence of the maximum copper concentration not inhibitory to growth, was analyzed by agarose gel electrophoresis, transfer to nitrocellulose, and hybridization to a CUP1-specific probe. The results of such experiments with 20 μg of RNA per lane are presented in Fig. 4. The chelatin

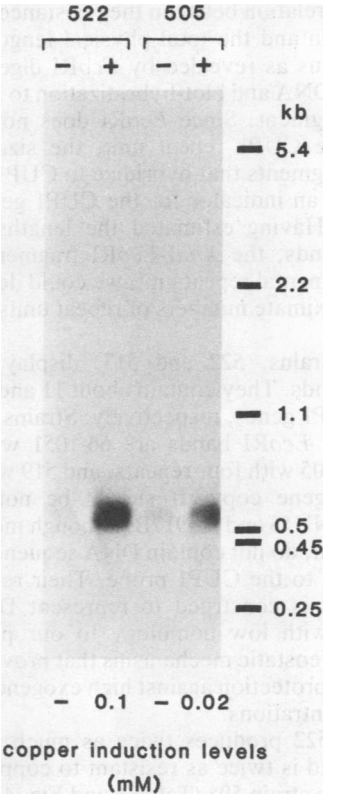

FIG. 4. DNA-RNA hybridization of mRNA from strains 522 and 505 (20 μg of RNA per lane) grown without (−) and with (+) added copper. Induction levels are given as concentration of copper (millimolar) at the bottom. Marker size and position are shown at the right.

mRNA level in the two strains is clearly regulated by exogenous copper. Based on visual inspection of autoradiograms, growth in copper-containing medium increases the chelatin mRNA level about 20-fold. This 20-fold increase in mRNA level is obtained reproducibly in duplicated trials involving diverse strains. Strain 522 produces about twice as much chelatin mRNA as strain 505, either in the presence or absence of added copper. The twofold increase in chelatin mRNA production in strain 522 compared with strain 505 is directly proportional to the twofold difference in CUP1 gene number and the twofold increase in copper resistance.

DISCUSSION

Various naturally occurring yeast strains, used extensively in commercial alcohol and wine production, differed considerably over a 20-fold range in their resistance to the inhibitory effects of copper on growth (Table 1). There is a

direct correlation between the resistance level of each strain and the total physical length of the CUP1 locus as revealed by *Eco*RI digestion of genomic DNA and blot-hybridization to a cloned CUP1 fragment. Since *Eco*RI does not cleave within the CUP1 repeat unit, the size of the *Eco*RI fragments that hybridize to CUP1 probes serves as an indicator for the CUP1 gene copy number. Having estimated the lengths of the *Eco*RI bands, the *Xba*I-*Eco*RI fragments, and the fundamental repeat unit, we could determine the approximate numbers of repeat units in each strain.

Two strains, 522 and 513, display double *Eco*RI bands. They contain about 11 and 2 and 9 and 5 CUP1 genes, respectively. Strains exhibiting single *Eco*RI bands are 66-1051 with nine repeats, 505 with four repeats, and 519 with only a single gene copy. It should be noted that strains YNN53 and 68-917B, although moderately resistant, do not contain DNA sequences with homology to the CUP1 probe. Their resistance to copper is construed to represent DNA sequences with low homology to our probe or other homeostatic mechanisms that provide considerable protection against high exogenous copper concentrations.

Strain 522 produces twice as much chelatin mRNA and is twice as resistant to copper compared with strain 505 (Table 1 and Fig. 4). Strain 513 is twice as resistant compared with strain 505, i.e., the same resistance level as strain 522 (the level of mRNA was not determined). Thus, the increased copper resistance observed among these commercial yeast strains appears to be intimately associated with increased CUP1 gene copy number and production of chelatin mRNA encoded by the CUP1 locus.

The analysis of the CUP1 locus in various *Saccharomyces* strains revealed the existence of both extensive and novel genetic diversity at the DNA structural and organizational levels. The primary variable concerns the number of CUP1 repeat units found in each strain. The number varies on a per chromosome basis from a single copy in strains JW251-1A and 519 to 10 or 11 copies in strains X2180-1A and 522. We emphasize that none of the strains analyzed was deliberately selected for copper resistance, including the two laboratory strains. However, by successive stepwise selection to higher copper resistance levels, we obtained derivative strains from X2180-1A. These strains contained a greater number of CUP1 genes than the progenitor X2180-1A (6). The existence of naturally occurring strains with high gene copy numbers for CUP1 and correspondingly high levels of copper resistance support current models that account for the origin of gene amplification (11). These models assume that (i) gene amplification arises through a spontaneous recombinational or replicational process and (ii) selection for an increased drug or metal resistance phenotype merely identifies those rare cells in which amplification has already occurred. Thus, selective pressures probably do not induce the amplification process, although in some instances they might stimulate it (11).

The present study does not address the variables that lead to the establishment of the amplified genotype in the various yeast strains examined. It is conceivable that during their long history as wine- or alcohol-producing fermentation agents, these strains were exposed to high copper ion levels as a result of repeated contact with Bordeaux mixture or with copper vessels. Hence, the occurrence of CUP1 gene amplification conferred a definite selective advantage upon their cells. The amplified genotype in any resistant strain is relatively stable, since it is maintained without any apparent selection, even over thousands of growth cycles, resulting in millions of tons of yeast biomass.

In addition, the precise molecular mechanisms responsible for maintaining sequence homogeneity among the tandemly iterated units are essentially unknown. In this connection, we may note that CUP1 is localized within a region of high positive chiasma interference (4, 7). This factor might contribute to the underlying common genetic mechanism responsible for both the observed sequence homogeneity and the extent to which tandemly iterated genes are amplified. Similar considerations apply to the rDNA locus of yeasts which is known to contain about 100 to 150 tandemly arrayed units of rDNA genes (8). Also, small but significant departures from ordinarily conversional parity (5) could readily provide sufficient drive, over time, to establish and maintain homogeneity among iterated sequences that experience intrachromosomal gene conversion (3). Further detailed structural and mutational analysis of the CUP1 locus may serve to elucidate the molecular basis for positive chiasma interference.

The various strains analyzed are quite diverse. They differ (i) in the number of the CUP1 genes they contain, (ii) in the size of the basic repeat unit, and (iii) in the number of CUP1 gene clusters. The basic CUP1 repeat unit in the laboratory strains is 2.0 kb (6), and the commercial strains, 505, 513, and 66-1051, carry a 1.5-kb repeat unit. In contrast, strain 522 contains repeat units of 1.5 and 1.7 kb (Fig. 3). How can we rationalize the existence of a variable repeat unit and constant junction fragments? Clearly, deletion within the repeat unit would shorten one or both *Xba*I-*Eco*RI junction fragments. Here, we propose a plausible mechanism for gene amplification. Starting with a single DNA

sequence, the model can generate iterations with 2.0, 1.7, or 1.5 kb. Our model (Fig. 5) assumes that within the 2.0-kb CUP1 locus there exist two or more short regions of homology identical to sequences immediately flanking the repeat unit. Oblique or misaligned pairing between homologous chromosomes or between sister chromatids is schematically described in Fig. 5. In Fig. 5a, paired homologous sequences are represented by small circles. A reciprocal crossover in this paired but misaligned region generates a tandem 2.0-kb repeat and retains the *Xba*I-*Eco*RI distances of 2.1 and 3.1 kb. If another homologous sequence exists 0.5 kb downstream from the *Kpn* site, it may pair as in Fig. 5b. A conventional exchange within the synaptic region generates a repeat of the 1.5-kb sequence that begins 0.5 kb to the right of the *Kpn*I site. The 0.5-kb portion is not duplicated. Again, the *Xba*I-*Eco*RI distances of 2.1 and 3.1 kb are retained. Similar arguments can be advanced to generate 1.7-kb repeats. Our model predicts the existence of a 2.0-kb band in a *Kpn*I digest as in Fig. 5a and no such fragment as in Fig. 5b. This prediction is fulfilled when we compare *Kpn*I digests of genomic DNA from strains 505 or 522 and X2180-1A (Fig. 6). Thus, *Kpn*I digests of X2180-1A contain a multimolar 2.0-kb band, whereas 505 and 522 digests display only long fragments indicative of cuts outside the 1.5-kb iterated region. Data to be published elsewhere indicate that the 1.5-kb repeat includes the external or flanking *Sau*3A site 0.1 kb to the right of the 2.0-kb repeat (see Fig. 1). Thus, to maintain a fit between the proposed model and the observed data, the paired homologous sequences (Fig. 5b) must be offset to the right by at least 0.1 kb. This argument would be strengthened if the 350-base-pair *Kpn*I-*Sau*3A fragment from the 2.0-kb repeat failed to hybridize with the 1.5-kb repeat.

The exact molecular basis for such differences is not entirely clear. However, we are currently engaged in cloning the CUP1 sequences from the Champagne yeast (strain 505) and the Montrachet strain (522). We are aware that not all of the 2.0-kb unit iterated in the copper-resistant strain X2180-1A is actually essential for the development of copper resistance. In fact, our unpublished studies demonstrate that transformation with autonomously replicating vectors carrying a small fragment of only 0.7 kb in length confers enhanced resistance on an otherwise sensitive cell.

The third level of diversity found in naturally occurring yeast strains concerns the number of CUP1 gene clusters, as revealed by *Eco*RI digestion of genomic DNA. Whereas the laboratory strain X2180-1A contains a single cluster of 10 repeat units, commercial strains 522 and 513 contain two clusters. These observations may be accounted for as follows. In some strains, a copy of the CUP1 locus might have been translocated or transposed to a nonhomologous chromosomal site. At the same time, an equivalent segment containing the original copies would be retained

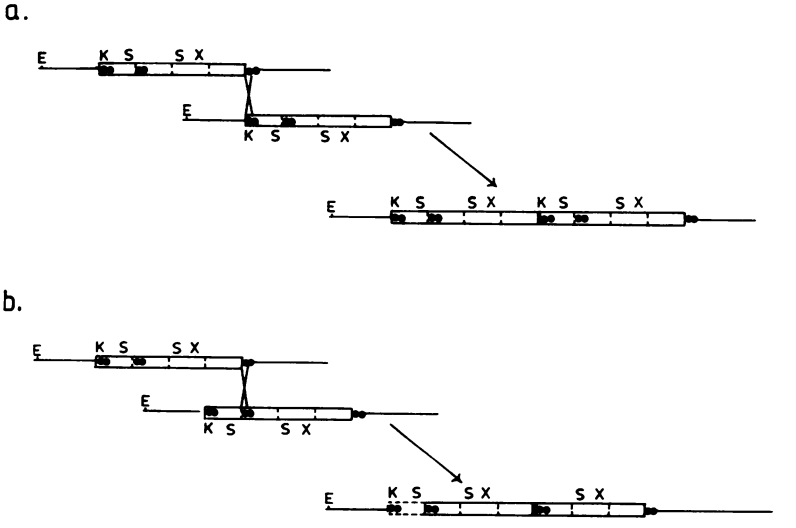

FIG. 5. A hypothetical model for generating two repeats from a single DNA sequence resulting in (a) two tandem 2.0-kb repeat units and (b) two tandem 1.5-kb repeat units. The large rectangle represents the 2.0-kb region which is iterated in the *CUP1*^{r} strains. It is divided into 0.5-kb sections (see Fig. 1 for complete map). Black circles depict hypothetical short homologous DNA sequences where crossing over might occur preferentially. Symbols: E, *Eco*RI; K, *Kpn*I; S, *Sau*3A; X, *Xba*I.

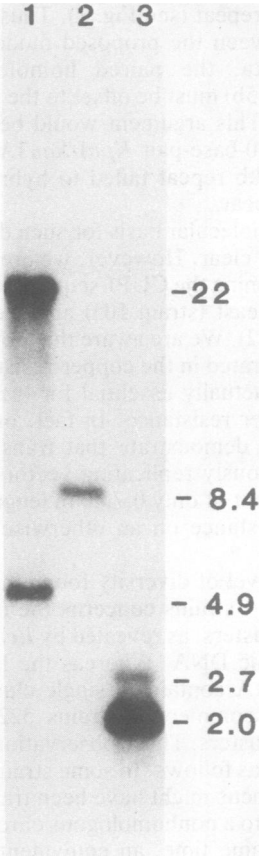

FIG. 6. DNA hybridization of a *Kpn* digest. Genomic DNA was isolated from strains 522 (lane 1), 505 (lane 2), and X2180-1A (lane 3). Band lengths in kb are given at the right.

at the original site. Alternatively, the strains displaying several CUP1 gene clusters might be variously aneuploid. The CUP1 segments may be of different length, and each may be carried on a different chromosome VIII. Thus, each chromosome VIII homolog would contain a CUP1 locus with a characteristic gene copy number. Accordingly, such aneuploid strains would be expected to generate several distinct *Eco*RI fragments. Strongly supportive of this view are our findings concerning a laboratory isolate P5A1, a strain exhibiting distinctly enhanced copper resistance. It was obtained through successive selections on media containing increasingly higher copper concentrations. When genomic DNA from this variant is subjected to *Eco*RI digestion and appropriately probed, two distinct electrophoretic bands are visualized. Both bands contain more than 10 iterated units characteristic of the X2180-1A progenitor strain. Moreover, when P5A1 is crossed to the single copy *cup1*^{s} strain, JW251-1A, and the resultant diploid is then subjected to tetrad analysis, as well as DNA hybridization analysis applied to the ascosporal clones, we find precisely those ascal segregation patterns expected for a trisomic diploid in which each homolog carries 11, 14, and 1 copies of the fundamental 2.0-kb repeat unit as well as the terminal junction segments (S. Fogel, J. W. Welch, G. Cathala, and M. Karin, Curr. Genet., in press). Thus, the gene copy number within the CUP1 locus is uniquely different on each of the segregating homologs. The development of two dissimilar CUP1 loci, each containing a different number of repeat units within a single cell, may be related to the phenomenon of positive chiasma interference. The recombination frequency between homologous chromosomes is markedly reduced in highly iterated gene clusters. In contrast, the recombination frequency between sister chromatids is increased (15). Increased unequal crossing over between sister chromatids and intrachromosomal gene conversion, along with depressed mitotic and meiotic recombination between homologs, could contribute to the origin and maintenance of gene clusters with different lengths. These may occupy the same position on the genetic map, although they are localized to individual copies of otherwise homologous chromosomes.

In summary, we identified a high degree of genetic diversity among naturally occurring industrial *Saccharomyces* strains used in commercial fermentation processes. These strains vary with respect to both the size and the copy number of the CUP1 locus. The naturally occurring amplification at this locus leads to overproduction of chelatin-mRNA and copper-binding protein and therefore to a higher degree of copper resistance. Gene amplification probably represents a series of spontaneous events rather than an effect induced by the selective agent.

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