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Caffeine Resistance of *Saccharomyces cerevisiae*

MARTIN BARD,* JEFFREY L. NEUHAUSER, AND NORMAN D. LEES

Department of Biology, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana 46205

Four caffeine-resistant haploid isolates, two resistant to 50 mM caffeine and two resistant to 100 mM caffeine, were genetically analyzed. Complementation and tetrad analysis indicated that all four mutations are alleles of the same locus. All four isolates demonstrated incomplete dominance when hybridized to the wild-type strain and dominance of high to low resistance when hybridized to one another. Differences in caffeine resistance were found between wild-type grande cells and its petite derivative.

Caffeine has been implicated as having a variety of biological effects in different organisms. Among these effects are cell killing, mutagenesis, and chromosome breakage in which caffeine has been demonstrated to cause or potentiate these effects or do both (7). For example, the frequencies of chromosomal aberrations produced in plant and animal cells by maleic hydrazide, UV irradiation, and alkylating agents are greatly increased by treatment with caffeine (8, 9). Another important biological effect of caffeine is that it increases the intracellular level of cyclic AMP by inhibiting the enzyme cyclic 3',5'-nucleotide phosphodiesterase (4).

In the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, caffeine has been demonstrated to be an inhibitor of a UV-induced recombinational repair process (5, 6). Loprieno et al. have demonstrated in *S. pombe* that caffeine present in genetic crossing medium may result in a 40 to 60% reduction in the number of meiotic recombinants (11). Hannan and Nasim have shown for *S. cerevisiae* that the minimum caffeine concentration required to observe caffeine-dependent UV sensitivity is a concentration which is lethal to 5 to 20% of unirradiated cells (6). Among four wild-type yeast strains, a haploid strain, S288C, was suggested to be caffeine resistant based upon its resistance to caffeine-induced killing and caffeine-dependent UV sensitivity.

This investigation was designed to elucidate the genetic aspects of caffeine resistance of *S. cerevisiae*. We also demonstrate that adenine in the growth medium partially protects wild-type yeast cells against the lethal effects of caffeine and that ethidium bromide-induced petite cells are more caffeine resistant than the parental wild-type grande cells.

Wild-type haploid strains of *S. cerevisiae* obtained from R. A. Woods (University of Winnipeg) were the parent **a** and α mating type strains used in this investigation and will hereafter be designated CA^+. These strains, previously designated su-pur^+, were derived from the Sheffield yeast collection and have been used as wild-type strains in studies of purine metabolism in yeast (1, 3, 10, 12, 13). The designation su-pur^+ indicates that this strain is wild type for a mutant strain, su-pur, which suppresses purine excretion. In this investigation, caffeine-resistant mutants were generated by ethyl methane sulfonate mutagenesis (50% kill) followed by screening on caffeine plates. After mutagenesis of ca. 1.5×10^8 CA^+ α cells, survivors were plated on a 1% yeast extract-2% peptone-2% dextrose-2% agar (YEPD) medium containing 10, 20, and 50 mM caffeine. Of the 21 isolates obtained, 4 will be discussed in this report: CA-5 isolated from a 10 mM caffeine plate, CA-10 and CA-18 isolated from 20 mM caffeine plates, and CA-20 isolated from a 50 mM caffeine plate. Table 1 shows the range of resistance of these four isolates and the wild-type strain CA^+. Resistance to caffeine is defined as confluent growth on 20 mM caffeine plates. CA-5 and CA-10 are the least-resistant isolates growing on 50 mM caffeine but not on 100 mM caffeine, whereas CA-18 and CA-20 are the most-resistant isolates growing well on 100 mM caffeine and spottedly on 120 mM caffeine.

The types of questions amenable to genetic analyses are the dominance-recessivity relationships, the allelic relationships, and the number of genetic loci involved. The levels of caffeine resistance from hybrids produced from crosses between the four resistant isolates and the wild-type strain are listed in Table 2. In no case were the hybrids fully as sensitive as the wild-type haploid or diploid strains and in no case were these hybrids as resistant as the original haploid resistant isolates. The more-resistant mutants, CA-18 and CA-20, when crossed to wild type produced hybrids that were more resistant than the hybrids produced by the less-resistant mutants, CA-5 and CA-10. Thus, the lower levels of resistance produced by these four hybrids sug-

TABLE 1. *Range of caffeine resistance levels of haploid mutant and wild-type S. cerevisiae*

Strain[a]	Resistance[b]						
	10[c]	20	40	50	100	120	150
CA-5	++	++	+	+	−	−	−
CA-10	++	++	+	+	−	−	−
CA-18	++	++	++	++	+	±	−
CA-20	++	++	++	++	+	±	−
CA^+	+	−	−	−	−	−	−

[a] Recorded 48 h after streaking on YEPD plates containing caffeine.
[b] ++, Heavy confluent growth; +, confluent but lighter growth; ±, spotted growth without confluence; −, no growth.
[c] Millimolar concentrations of caffeine.

TABLE 2. *Caffeine resistance determinations of hybrids*

Hybrid[a]	Resistance[b]						
	10[c]	20	40	50	100	120	150
CA-5/CA^+	++	±	−	−	−	−	−
CA-10/CA^+	++	±	−	−	−	−	−
CA-18/CA^+	++	++	±	−	−	−	−
CA-20/CA^+	++	++	+	−	−	−	−
CA^+/CA^+	+	−	−	−	−	−	−
CA-5/CA-10	++	++	+	+	−	−	−
CA-5/CA-18	++	++	+	+	±	±	−
CA-5/CA-20	++	++	+	+	±	±	−
CA-10/CA-18	++	++	+	+	±	±	−
CA-10/CA-20	++	++	++	+	±	±	−
CA-18/CA-20	++	++	++	+	+	±	−

[a] Recorded 48 h after streaking on YEPD plates containing caffeine. Slash (/) denotes haploids crossed to produce hybrid strain. Hybridization and ascus dissection have been described (2).
[b] ++, Heavy confluent growth; +, confluent but lighter growth; ±, spotted growth without confluence; −, no growth.
[c] Millimolar concentrations of caffeine.

gest that these CA mutants demonstrate incomplete dominance when crossed to the wild type. The resistance levels of hybrids made from pairwise crosses of mutants are also listed in Table 2. In each case all hybrids showed a resistance level equal to that of the more highly resistant strain, indicating an absence of complementation and therefore suggesting allelism. Thus, CA-5 and CA-10 carried low-resistance alleles and behaved as recessive strains when hybridized to the strains carrying high-resistance alleles, CA-18 and CA-20.

Segregation analyses of these diploids confirm and extend the above conclusions as follows. (i) In each case in which a mutant was hybridized to the wild-type strain, single gene segregation was observed in that each cross yielded two resistant and two sensitive ascosporal colonies. The numbers of asci tested and the level of caffeine resistance in millimolar concentrations which resulted in these ratios are listed in Table 3. (ii) The segregation pattern of mutant × mutant hybrids always yielded a 4:0 (resistant to

TABLE 3. *Tetrad analysis of caffeine-resistant hybrids*

Hybrid[a]	No. of asci	Segregation ratio
CA-5/CA^+	21	2:2[b] (40)[c]
CA-10/CA^+	18	2:2 (40)
CA-18/CA^+	28	2:2 (40); 2:2 (100)
CA-20/CA^+	17	2:2 (40); 2:2 (100)
CA-5/CA-10	11	4:0 (50);
CA-5/CA-18	12	4:0 (50); 2:2 (100)
CA-5/CA-20	12	4:0 (50); 2:2 (100)
CA-10/CA-18	16	4:0 (50); 2:2 (100)
CA-10/CA-20	11	4:0 (50); 2:2 (100)
CA-18/CA-20	9	4:0 (100)

[a] Slash (/) denotes haploids crossed to produce hybrid strain.
[b] Ratio of resistant to sensitive ascospores.
[c] Numbers in parentheses indicate caffeine concentration (millimolar) which resulted in the given segregation ratio.

sensitive) pattern on low levels of caffeine (40 mM). At higher levels of caffeine (100 mM) a 2:2 or 4:0 resistant-to-sensitive segregation pattern was observed, depending upon whether the hybrid was composed of a low- and a high-resistant haploid or both highly resistant haploids, respectively. No wild-type segregants were observed among the 71 asci analyzed from mutant × mutant hybrids, and these results, together with the absence of complementation, suggest that CA-5, -10, -18, and -20 are alleles.

Spontaneously derived caffeine-resistant mutants were also observed by plating ca. 10^4 cells on 10, 20, and 50 mM caffeine plates. After 2 weeks, 15 colonies were isolated from the 10 and 20 mM caffeine plates. Of the 15 colonies isolated, 6 showed spotted or heterogeneous growth upon restreaking. Further analysis of these six isolates indicated that within these heterogeneous populations the slower-growing cells were both caffeine resistant and respiratory deficient (petite)—failing to grow when ethanol or glycerol was used as the carbon source. Attempts to cross these petite isolates to the wild-type strain resulted in diploids that were likewise petite, and thus the original petite isolates were presumed to be suppressive petite isolates. Testing of the grande isolates (from the heterogeneous populations and the nine homogeneous colonies) on caffeine media resulted in nonresistance.

To further investigate the relationship between respiratory deficiency and caffeine resistance, we treated wild-type CA^+ cells with ethidium bromide to induce the respiratory-deficient (petite) phenotype (14). Wild-type and petite strains were grown overnight on a 0.67% yeast nitrogen base plus 2% dextrose (YNBD) medium. Samples were collected by centrifugation, washed twice in 5 ml of purine supplemented

TABLE 4. *MGT of a wild-type and petite mutant in caffeine- and purine-supplemented media*

Strain	MGT (h)						
	YNBD[a] alone	10 mM adenine	10 mM caffeine	5 mM adenine + 10 mM caffeine	10 mM adenine + 10 mM caffeine	20 mM adenine + 10 mM caffeine	20 mM guanine + 10 mM caffeine
Wild-type grande	1.8[b] (1.0)[c]	1.7 (0.94)	14.5 (8.1)	10.7 (5.9)	8.9 (4.9)	9.2 (5.1)	15.0 (8.3)
Wild-type petite	3.4 (1.0)	3.2 (0.94)	10.4 (3.1)	10.8 (3.2)	10.9 (3.2)	10.5 (3.1)	10.3 (3.0)

[a] All media contained YNBD.
[b] Average of duplicate samples.
[c] Numbers in parentheses indicate the fold increase as compared to cells grown in unsupplemented YNBD medium.

YNBD, and suspended in a final volume of 20 ml in a nephelometer flask. The initial concentration of all samples was 1 to 5 Klett units (no. 66 red filter, ca. 4.5×10^5 to 2.25×10^6 cells per ml). Readings were taken periodically, and the mean generation time (MGT) was calculated.

Table 4 lists the growth responses of the wild-type grande strain CA^+ and the derived petite mutant. The MGT of the petite strain was essentially twice that of the grande strain. The addition of 10 mM caffeine to the medium resulted in an eightfold increase in MGT for the grande strain and a threefold increase for the petite strain. The addition of adenine to caffeine-supplemented media resulted in a partial growth-sparing effect for the grande strain. A concentration of 10 mM adenine was the most effective, reducing the MGT increase from eight- to fivefold. However, adenine did not exhibit a growth-sparing effect with the petite strain in caffeine-supplemented media. The adenine growth-sparing effect was not the result of its functioning as a nutrient source because adenine-supplemented medium (YNBD plus adenine) did not significantly promote more efficient growth in either the grande or petite strains compared to that observed in YNBD alone. The addition of 20 mM guanine to a caffeine-supplemented medium resulted in no growth-sparing effect for either the grande or petite strain. Although we have no explanation for the increased resistance of the petite strain over that of the grande, it is not unlikely that the caffeine-sensitive component in the grande strain is related to its capacity for respiratory adaptation.

Several possibilities exist as to the molecular basis of caffeine resistance. First, there might be a change in enzyme specificity such that caffeine is not incorporated into one or more essential cell metabolites. For example, caffeine toxicity may be due to its conversion to inhibitory adenine analogs of ATP, NAD^+, or FAD^+. Second, the cell may acquire an increased ability to chemically modify caffeine, resulting in an amelioration of its toxic effects. Third, membrane permeability to caffeine may be changed due to the alteration of a permease.

Pickering and Woods (12) have isolated seven yeast mutants resistant to the purine analog, 4-aminopyrazolo(3,4-d)pyrimidine (4-APP). One of these mutants, *app1-14*, exhibited decreased uptake of adenine and hypoxanthine, but no detectable uptake of 4-APP. These investigators concluded that there are at least two purine permeases in yeast: an adenine permease which also accepts hypoxanthine, 4-APP, and other analogs and a guanine permease which also accepts hypoxanthine. The nature of the *app1-14* lesion is explained by an alteration in the adenine permease which excludes 4-APP but still accepts adenine as a substrate. The guanine permease is unchanged (13). Among three *app* mutants tested for caffeine resistance (*app1-14*, *app4-18*, and *app5-24*) only *app1-14* was found to be slightly caffeine resistant (± on 20 mM caffeine). Unfortunately, the level of resistance was too low to accurately test for allelism with the CA mutants described here. Other purine mutants isolated by Burridge et al. (3) which excrete purines into the growth medium were also found to be definitely caffeine sensitive. If caffeine is predominantely transported into the yeast cell by means of an adenine permease, this might help to explain caffeine resistance (the exclusion of caffeine by the permease), the adenine growth-sparing effect (competition between adenine and caffeine for entry), and the virtual lack of a guanine growth-sparing effect (due to guanine being transported by a unique permease).

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