Genetic and Biochemical Properties of Thialysine-resistant Mutants of *Saccharomyces cerevisiae*

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Three groups of lysine-excreting, thialysine-resistant mutants of *Saccharomyces cerevisiae* were derived from the wild-type strain (X2180) by mutagenic treatment and selected on the basis of a cross-feeding assay. Mutants MNNG2-9, MNNG2-27, MNNG2-39 and MNNG2-62 (group I) exhibited a 2:2 segregation for thialysine resistance following mating with a wild-type strain and a lower than wild-type lysyl-tRNA synthetase activity; the thialysine-resistant phenotype was dominant in specific hybrids. Mutant MNNG2-2 (group II) was similar to group I mutants except that the thialysine-resistant phenotype was recessive in the hybrid. Mutant MNNG3-142 (group III) exhibited an irregular ratio of segregation of thialysine resistance and a significantly lower lysyl-tRNA synthetase activity; the thialysine-resistant phenotype was recessive in the hybrid. The growth of both group I and group III mutants was temperature-sensitive. The thialysine-resistant mutants exhibited pleiotropic properties including the increased production and excretion of lysine, thermosensitive growth and an impairment of lysyl-tRNA synthetase activity.

INTRODUCTION

Metabolite analogues have been used extensively to study the mechanism of regulation of the biosynthesis of amino acids in bacteria (Umbarger, 1971) and to a lesser extent in lower eukaryotic organisms including yeast (Delforge *et al.*, 1975; Greer & Fink, 1976; Hutter, 1979). Thialysine, an analogue of lysine, inhibits the growth of several micro-organisms including *Saccharomyces cerevisiae* (Coles & Brenchley, 1976; Freidrich & Demain, 1977; Haidaris & Bhattacharjee, 1978). Thialysine-resistant mutants exhibit altered lysyl-tRNA synthetase activity in *Escherichia coli* (Hirschfield & Zamechnik, 1971), altered regulation of lysine-sensitive aspartokinase in *Bacillus subtilis* and *Salmonella typhimurium* (Vold *et al.*, 1975; Jegede *et al.*, 1976) and an increased production and excretion of lysine in *Brevibacterium flavum*, *Candida pelliculosa* and *S. cerevisiae* (Sano & Shiio, 1970; Takenouchi *et al.*, 1977; Haidaris & Bhattacharjee, 1978). Since most cereals are deficient in lysine, high lysine-excreting mutants of bacteria and yeast are of special interest in the production of lysine. Also, an increased production of lysine in such mutants would suggest an impairment of the normal regulation of the biosynthesis of lysine.

In yeast and other fungi there are fewer mutants available for the study of aminoacyl-tRNA synthetase than in bacteria (Neidhardt *et al.*, 1975) and the role of specific aminoacyl-tRNA synthetases in the regulation of the biosynthesis of the corresponding amino acids is not well understood. Since the pathway for the biosynthesis of lysine in *S. cerevisiae* is completely different from the pathway used by bacteria (Broquist & Trupin, 1966; Bhattacharjee & Sinha, 1972), the effects of thialysine-resistant mutants on the biosynthesis of lysine in this organism are of special interest. We report here for the first time the genetic and biochemical properties of some lysine-excreting, thialysine-resistant mutants of *S. cerevisiae*.

METHODS

Strains. Wild-type strains X2180-1A (*a thl*) (courtesy of Dr R. K. Chan) and 609 (*α thl*) (courtesy of Dr C. C. Lindégren), lysine-producing thialysine-resistant mutants of *Saccharomyces cerevisiae* (Table 1), and a lysine auxotroph *lys3* of *Rhodotorula glutinis* MU-1 (Kinzel & Bhattacharjee, 1979) were used.

Media and growth conditions. Nutrient medium (Gray & Bhattacharjee, 1976) was used for the growth of colonies after mutagenic treatment and to maintain stock cultures. Minimal medium was used for the selection of lysine-producing mutants. Minimal agar medium (containing: Difco yeast nitrogen base without amino acids and ammonium sulphate, 6.7 g; thialysine, 0.1 g; glucose, 10 g; proline, 0.5 g; agar, 25 g; distilled water, to 1 l) was used for the selection and maintenance of thialysine-resistant mutants. Sporulation medium was the same as that described by McClary *et al.* (1959). All cultures were incubated at 30 °C, unless otherwise indicated, and those in liquid medium were shaken.

Selection of lysine-excreting, thialysine-resistant mutants. Mutagenic treatment and the screening of thialysine-resistant mutants were performed according to procedures described previously (Haidaris & Bhattacharjee, 1978), and the lysine-excreting mutants were selected on the basis of a cross-feeding assay. A lawn was prepared with 0.1 ml suspension (about 1×10^5 cells ml⁻¹) of *R. glutinis* lysine auxotroph *lys3* on each plate of minimal agar medium and the plates were incubated for several hours to allow the surface to dry. Six or seven thialysine-resistant mutants and the parent wild-type strain were then seeded on each plate and the plates were incubated for 5 to 10 d. Thialysine-resistant mutants which supported growth of the *R. glutinis* lysine auxotroph by cross-feeding were selected as high lysine-producing, thialysine-resistant mutants (Fig. 1). Red colonies of *R. glutinis* lysine auxotroph resulted from the cross-feeding, thus eliminating any doubt as to the excess lysine-producing and lysine-excreting ability of the thialysine-resistant mutants.

Genetic analysis. Mating, sporulation, micromanipulation and tetrad analysis were performed according to standard procedures (Sherman & Lawrence, 1974). Hybrids were prepared by mating each mutant with wild-type strain 609. The dominance of thialysine resistance was determined in diploid cells isolated by micromanipulation following mating of a thialysine-resistant mutant with wild-type strain 609. Each colony from isolated single cells was examined for diploidy on the basis of the ability to sporulate in sporulation medium and for thialysine resistance by the ability to grow on thialysine-supplemented minimal agar medium.

Lysyl-tRNA synthetase activity. Cells from 62 h cultures were disrupted using a Bronwill MSK homogenizer (Bronwill Scientific Co., San Mateo, Calif., U.S.A.) (Gray & Bhattacharjee, 1976). Cell extracts were dialysed against 100 vol. 0.08 M-Tris/HCl buffer pH 7.3 in the cold for 18 h with three changes of buffer. Protein was determined by the biuret method using bovine serum albumin as a standard (Gornall *et al.*, 1949). The reaction mixture for the assay of lysyl-tRNA synthetase [L-lysine:tRNA^{Lys} ligase (AMP-forming); EC 6.1.1.6] activity contained (in 1 ml): purified yeast tRNA (Sigma type X or type 1), 45 units; Tris/HCl buffer pH 7.3, 80 μ mol; MgCl₂, 7.5 μ mol; ATP, 4 μ mol; reduced glutathione, 3 μ mol; L-[U-¹⁴C]lysine, 2.56 nmol; cell extract, equivalent to 2 mg protein. The ATP was neutralized to pH 7.0 with KOH before addition. The mixture was incubated in a water bath at 37 °C for 15 min and the reaction was stopped by adding 4 ml cold 5% (w/v) trichloroacetic acid

Table 1. *Strains of Saccharomyces cerevisiae**

Strain	Genotype
X2180-1A	<i>a thl</i>
609	<i>α thl</i>
MNNG2-2	<i>a THL</i>
MNNG2-3	<i>a THL</i>
MNNG3-142	<i>a THL</i>
MNNG2-9	<i>a THL</i>
MNNG2-7	<i>a THL</i>
MNNG2-13	<i>a THL</i>
MNNG2-27	<i>a THL</i>
MNNG2-39	<i>a THL</i>
MNNG2-52	<i>a THL</i>
MNNG2-60	<i>a THL</i>
MNNG2-62	<i>a THL</i>
MNNG2-69	<i>a THL</i>
MNNG2-72	<i>a THL</i>
MNNG27-1-31	<i>α THL</i>
MNNG27-1-12	<i>α THL</i>
MNNG27-31-9-17	<i>α THL</i>

* Thialysine-resistant (*THL*) strains were developed in this project. Sources of the wild-type strains (X2180-1A and 609) are given in Methods.

and keeping the tube in ice for 10 min. The reaction mixture was filtered through a cellulose filter (0.45 μ m pore diam.). The filters were washed three times with 4 ml cold trichloroacetic acid solution. The radioactivity associated with each filter was measured using a toluene/ethanol scintillation fluid in a Beckman LS100 liquid scintillation spectrometer. The control lacked ATP.

Chemicals. Fine chemicals were from Sigma, media were from Difco, L-[U- 14 C]lysine was from ICN Chemical & Radioisotope (Irvine, Calif., U.S.A.) and glucosylase was from Endo Laboratories (Garden City, N.Y., U.S.A.).

RESULTS

Lysine-producing, thialysine-resistant mutants

After mutagenic treatment of wild-type strain X2180-1A with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 14 450 colonies were examined of which 418 were thialysine-resistant. A total of 37 high lysine-producing, thialysine-resistant colonies were selected on the basis of the cross-feeding assay (Fig. 1). Chemical and microbiological assays were used for the determination of lysine. Each of these mutants produced at least 100 μ g lysine per ml culture. Wild-type cells neither accumulate lysine in the intracellular pool nor excrete any significant amount of lysine compared with the mutant strains (Haidaris & Bhattacharjee, 1978).

Determination of the segregation ratio for selected thialysine-resistant mutants

To determine whether or not the thialysine-resistant phenotype of the lysine-producing mutants was due to the mutation of a single nuclear gene, tetrads from individual hybrids between specific mutants and wild-type strain 609 (α , *thl*) were analysed (Table 2). Five of the mutants (MNNG2-2, MNNG2-9, MNNG2-27, MNNG2-39 and MNNG2-62) exhibited 2:2 segregation for thialysine resistance and mating-type. Two of these mutants (MNNG2-9 and MNNG2-39) exhibited one tetrad each with a 3:1 segregation for thialysine resistance. Tetrad distributions between the mating-type locus and thialysine resistance indicated no significant trend for any type of linkage. Highly irregular segregation was observed for thialysine resistance in all tetrads from hybrids between mutants MNNG2-3 and MNNG3-142 with strain 609. A second generation backcross of mutant MNNG3-142 also

Fig. 1. Selection of lysine-excreting, thialysine-resistant mutants on the basis of cross-feeding assay. A lawn of *R. glutinis* lysine auxotroph *lys3* was prepared on a minimal agar plate and seven thialysine-resistant mutants and the wild-type strain X2180-1A (negative control in the centre of the plate) were seeded on to the agar surface. After several days of incubation, high lysine-producing mutants were selected on the basis of their ability to support the growth of the *R. glutinis* lysine auxotroph (small colonies).

Table 2. *Determination of the segregation ratio of the mating type and thialysine resistance and the tetrad distributions*

Hybrid	Tetrads exhibiting 2:2 segregation for		Tetrads exhibiting 3:1 segregation for <i>THL:thl</i>	Tetrad distributions* for <i>a-THL</i>		
	<i>THL:thl</i>	<i>a:a</i>		PD	NPD	TT
MNNG2-2 (<i>a THL</i>) × 609 (<i>a thl</i>)	10	10	0	1	0	9
MNNG2-9 (<i>a THL</i>) × 609 (<i>a thl</i>)	16	17	1 (3:1)	3	4	10
MNNG2-27 (<i>a THL</i>) × 609 (<i>a thl</i>)	16	16	0	2	3	11
MNNG2-39 (<i>a THL</i>) × 609 (<i>a thl</i>)	19	20	1 (3:1)	4	5	11
MNNG2-62 (<i>a THL</i>) × 609 (<i>a thl</i>)	13	13	0	1	3	9

* PD, Parental ditype; NPD, non-parental ditype; TT, tetratype.

Table 3. *Determination of dominance or recessiveness of the thialysine-resistant phenotype for selected mutants*

Hybrid	No. of diploid isolates tested	Growth on thialysine medium	Sporulation	Dominant or Recessive
MNNG2-9 (<i>a THL</i>) × 609 (<i>a thl</i>)	4	+	+	Dom.
MNNG2-2 (<i>a THL</i>) × 609 (<i>a thl</i>)	2	—	+	Rec.
MNNG3-142 (<i>a THL</i>) × 609 (<i>a thl</i>)	4	—	+	Rec.
MNNG2-27 (<i>a THL</i>) × 609 (<i>a thl</i>)	5	+	+	Dom.
MNNG2-39 (<i>a THL</i>) × 609 (<i>a thl</i>)	5	+	+	Dom.
MNNG2-62 (<i>a THL</i>) × 609 (<i>a thl</i>)	5	+	+	Dom.

Table 4. *Recombination analyses among some thialysine-resistant mutants*

Hybrids were constructed between specific thialysine-resistant mutants. Viable segregants (tetrads and individual spores) were tested for the ability to grow in minimal medium and minimal medium containing thialysine.

Hybrid	Tetrad distribution*			Total no. of spores	No. of spores recombined
	PD	NPD	TT		
MNNG2-2 (<i>a THL</i>) × 24C (<i>a THL</i>)†	6	3	7	177	37
MNNG2-62 (<i>a THL</i>) × 24C (<i>a THL</i>)				46	5
MNNG2-27 (<i>a THL</i>) × 9a (<i>a THL</i>)‡	2	4	3	128	38
MNNG2-62 (<i>a THL</i>) × 9a (<i>a THL</i>)	1	1	0	115	15
MNNG2-39 (<i>a THL</i>) × 9a (<i>a THL</i>)				129	28

* PD, Parental ditype; NPD, non-parental ditype; TT, tetratype.

† Segregant of MNNG2-9 (*a THL*) × 609 (*a thl*).‡ Segregant of MNNG2-2 (*a THL*) × 609 (*a thl*).

exhibited irregular segregations (results not shown). Several other mutants were not amenable to genetic analysis because of the lack of ability to mate and/or sporulate.

Dominance or recessiveness of the thialysine-resistant phenotype

Colonies from isolated single diploid cells of hybrids between wild-type strain 609 and mutants MNNG2-9, MNNG2-27, MNNG2-39, or MNNG2-62 grew on thialysine-supplemented minimal agar medium within 72 h. These isolates also sporulated confirming their diploid state (Table 3). In the case of mutants MNNG2-2 and MNNG3-142, the isolated heterozygous diploids did not grow on thialysine-supplemented minimal agar medium. Ascospores from one of these diploids showed 12 thialysine-resistant and 11 thialysine-

Table 5. *Lysyl-tRNA synthetase activity of thialysine-resistant mutants*

Strain	Lysyl-tRNA synthetase activity (% of wild type)*
X2180-1A (<i>a thl</i>)	100
MNNG2-2	65
MNNG3-142	33
MNNG2-9	74
MNNG2-27	57
MNNG2-39	78
MNNG2-62	48
MNNG2-7	91
MNNG2-13	44
MNNG2-52	95
MNNG2-60	50
MNNG2-69	39
MNNG2-72	65
MNNG27-1-12	21
MNNG27-1-31	38
MNNG27-31-9-17	32

* The wild-type activity was 0.24 nmol L-lysine charged (mg protein)⁻¹ h⁻¹.

Table 6. *Growth response of thialysine-resistant mutants at 30 and 37 °C*

Test tubes containing 8 ml minimal medium were inoculated with 0.1 ml cell suspension (A_{550} 0.5) and incubated with gentle shaking at either 30 or 37 °C. A_{550} was measured at the times indicated.

Strain	Absorbance at 550 nm				
	Growth at 30 °C		Growth at 37 °C		
	13 h	38 h	13 h	38 h	45 h
X2180-1A	0.07	>2	0.08	>2	>2
MNNG2-2	0.03	>2	0.08	>2	>2
MNNG2-3	0.10	>2	0.15	>2	>2
MNNG3-142	0.09	>2	0.13	0.10	0.11
MNNG2-9	0.02	>2	0.02	0.12	0.12
MNNG2-27	0.05	>2	0.05	0.05	0.04
MNNG2-39	0.06	>2	0.07	0.10	0.11
MNNG2-62	0.09	>2	0.04	0.06	0.06

sensitive (1 : 1 ratio) segregants indicating the heterozygous nature of the isolated diploid cells.

Several crosses were made among the mutants or their progeny. Because of poor germination, conventional tetrad analysis for the determination of linkage among the mutants was not possible. Recombinants were obtained from a small number of tetrads and isolated single spores from all of the hybrids were tested (Table 4).

Lysyl-tRNA synthetase activity in selected mutants

The lysyl-tRNA synthetase activity was determined in a number of thialysine-resistant mutants; activities ranged between 21 and 95 % of the wild-type activity (Table 5). Of the 15 mutants examined, seven showed less than 50 % and seven more than 50 % of the activity of the parent strain.

Temperature sensitivity of thialysine-resistant mutants

Several thialysine-resistant mutants were examined for their ability to grow at 37 °C (Table 6). As expected, all of the mutants grew well at 30 °C, but only two mutants (MNNG2-2 and MNNG2-3) and the wild-type strain grew at 37 °C. The remaining mutants showed no significant growth at 37 °C even after 45 h.

DISCUSSION

The ability of thialysine-resistant mutants to produce excess lysine per unit of culture compared with the wild-type strain is an indication that the regulation of the biosynthesis of lysine has been impaired in such mutants. Therefore, in an attempt to obtain possible regulatory mutants for the biosynthesis of lysine, only lysine-producing, thialysine-resistant mutants were isolated and used in this study for genetic and biochemical characterization. The majority of the thialysine-resistant mutants, however, did not produce lysine. Since the parent wild-type strain is sensitive to thialysine, it is assumed that mutants that did not excrete lysine were resistant to thialysine because of the inability to transport the analogue into the cells as a result of mutation (Umbarger, 1971). Some of these mutants may still, however, be regulatory mutants for the biosynthesis of lysine.

A 2:2 segregation for thialysine resistance in the tetrads demonstrates that the thialysine-resistant phenotype in each of the mutants MNNG2-2, MNNG2-9, MNNG2-27, MNNG2-39 and MNNG2-62 is the result of a single nuclear gene mutation. A 3:1 segregation for thialysine resistance in the case of mutants MNNG2-9 and MNNG2-39 can be explained on the basis of gene conversion (Lindgren, 1953; Mortimer & Hawthorne, 1976). A predominantly 2:2 segregation with a low frequency of 3:1 or 1:3 segregation is commonly observed when a phenotype is due to a single nuclear gene mutation. An irregular segregation ratio for thialysine resistance indicates that the mutants MNNG2-3 and MNNG3-142 contain more than one nuclear gene mutation.

Results of the dominance test indicate that more than one gene is responsible for thialysine resistance in these mutants. This conclusion is based on the observation that four of the mutants (MNNG2-9, MNNG2-27, MNNG2-39 and MNNG2-62) express their resistance as dominant and two (MNNG2-2 and MNNG3-142) express their resistance as recessive. None of the mutants tested are linked to the mating-type locus based on the ratio of PD, NPD and TT tetrads. Two of the mutants (MNNG2-9 and MNNG2-39) may be linked to a centromere other than the one in chromosome III which contains the centromere-linked mating-type locus (Mortimer & Hawthorne, 1976). Preliminary results from recombination analysis involving several mutants indicate that the thialysine-resistant mutations are not linked to each other and that more than one gene is likely to be responsible for thialysine resistance.

Different activities of lysyl-tRNA synthetase were present in different mutants. Several mutants showed less than 50% of the wild-type activity. These differences in activity suggest that the selected mutants have undergone different mutations either in the same gene or in different genes which affect the synthesis or the activity of lysyl-tRNA synthetase. Hirschfield & Zamechnik (1971) have reported thialysine-resistant mutants of *E. coli* with 5 to 50% of the wild-type lysyl-tRNA synthetase activity. In bacteria, only one lysyl-tRNA synthetase is known and this activity from *E. coli* is inhibited by thialysine (Stern & Mehler, 1965). Genetic and regulatory properties of lysyl-tRNA synthetase of *S. cerevisiae* are not known (Rymo *et al.*, 1972).

Thialysine-resistant mutants in bacteria elicit pleiotropic effects including alteration of the quality (feedback sensitivity) and the quantity (repressibility) of specific biosynthetic enzymes as well as the impairment of lysyl-tRNA synthetase activity (Hirschfield & Zamechnik, 1971; Coles & Brenchley, 1976). The role of tRNA synthetase mutations on the derepression of biosynthetic enzymes is well known in bacteria (Neidhardt *et al.*, 1975). A derepression of the isoleucine, methionine and threonine biosynthetic enzymes in their corresponding tRNA synthetase mutants is also known in *S. cerevisiae* (McLaughlin *et al.*, 1969; Nass & Hasenbank, 1970; Cherest *et al.*, 1975). Such a derepression of biosynthetic enzymes coupled with the feedback insensitivity of homocitrate synthase (Gray & Bhattacharjee, 1976; Heslot *et al.*, 1979) would explain the increased production and excretion of lysine in specific mutants. Results from the studies on dominance, recombination, temperature sensitivity and lysyl-tRNA synthetase activity suggest that more than one (possibly three) nuclear gene is involved in the determination of thialysine resistance and lysyl-tRNA synthetase activity in *S. cerevisiae*.

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