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Mechanism of resistance to sulphite in *Saccharomyces cerevisiae*

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Abstract. Growth inhibition and cell killing caused by sulphite were reduced in seven *Saccharomyces cerevisiae* sulphite-resistant independent mutants, compared to their parental strains. Genetic analysis showed that in the seven mutants resistance was inherited as a single-gene dominant mutation and that all the analyzed mutations were allelic, thus identifying a major gene responsible for sulphite resistance in *S. cerevisiae*. Two of the mutants, MBS20-9 and MBS30, were further characterized. ^{35}S-sulphite uptake experiments showed that the ability to accumulate sulphite was markedly reduced in the two resistant strains. No difference between resistant and sensitive strains with respect to glyceraldehyde-3-phosphate dehydrogenase sensitivity to sulphite, or to intracellular glutathione content, were revealed. In contrast, the extracellular acetaldehyde concentration was higher in the resistant mutants, both in the presence and in the absence of sulphite.

Key words: Sulphite-resistant mutants – Sulphite uptake – Acetaldehyde accumulation – *Saccharomyces cerevisiae*

Introduction

It has been known for a long time that sulphite acts as a powerful antimicrobial agent. It dissolves in water leading to the formation of bisulphite (HSO_3^-), sulphite (SO_3^{2-}), and sulphur dioxide (SO_2). All these readily interchangeable sulphur species are usually referred to as 'sulphite'. They exist, in solution, in a pH-dependent equilibrium as follows:

$$SO_2 + H_2O \rightleftharpoons HSO_3^- + H^+ \rightleftharpoons SO_3^{2-} + H^+.$$

An increase in acidity shifts the equilibrium to the left with a concomitant increase in the antimicrobial action of sulphite. This phenomenon has been explained by the identification of SO_2 as the active agent (Macris and Markakis 1974; Schimz 1980). Sulphite is particularly active when added to yeast culture media with pH values in the range of 3.0–5.0 (Rose 1987). Moreover, among the three molecular forms of sulphite, only SO_2 enters the yeast cell (Macris and Markakis 1974; Stratford and Rose 1986). The transport mechanism could be an active carrier-mediated process (Macris and Markakis 1974) or else simple diffusion is involved (Stratford and Rose 1986). Once inside the cell, sulphite can react with a number of cellular molecules (Shapiro 1977; Gunnison 1981) resulting in important effects on energy metabolism (Schimz 1980; Hinze and Holzer 1986) and cytoplasmic pH (Pilkington and Rose 1988).

However, the relative role of the various sulphite reactions in yeast killing and growth inhibition remains to be evaluated. Similarly, little is known about the physiological basis of sulphite resistance in yeast. Stratford et al. (1987) proposed that the ability to produce carbonyl compounds, particularly acetaldehyde, could explain differences in sulphite resistance among different species of yeast. They also speculated that differences in sulphite resistance among yeasts could be due either to different rates of sulphite transport or a different ability to accumulate it. A possible role of glutathione levels and of glutathione reductase activity in sulphite detoxification has been proposed in the rat (Kagedal et al. 1986), plants (Grill et al. 1982; Chiment et al. 1986) and yeast (Scardovi 1952; Casalone et al. 1989).

In the present paper we have further characterized previously isolated sulphite-resistant mutants of *S. cerevisiae* (Casalone et al. 1989). The cell permeability to sulphite, the level of sulphite binding compounds, and the glyceraldehyde-3-phosphate dehydrogenase sensitivity to sulphite were investigated in two independent mutants and in the sensitive parental strain. An allelism test on these and five other resistant mutants is also reported.

Materials and methods

Yeast strain and growth conditions. The *S. cerevisiae* strains used are listed in Table 1. Yeasts were cultured aerobically at 28 °C in YEPD

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Table 1. Yeast strains used

Strain	Genotype	M.I.C.[a] (mM sulphite)	Origin
S288C	*MATα*, *sul*^{S}[b]	1.2	S. Sora (Pavia University)
1425	*MATa*, *sul*^{S}, *ade1*, *his1*	1.2	S. Sora (Pavia University)
BD-4a	*MATa*, *sul*^{S} *can1*, *leu2-3*, *112* *trp1-289*, *ura3-52*	1.2	C.V. Bruschi (ICGEB Trieste)
MBS30	*MATα*, *SUL*^{R}[b]	3.6	Spontaneous mutant from S288C
MBS20-7	*MATα*, *SUL*^{R}	3.6	UV light-induced mutant from S288C
MBS20-9	*MATα*, *SUL*^{R}	3.6	UV light-induced mutant from S288C
MBS20-10	*MATα*, *SUL*^{R}	4.0	UV light-induced mutant from S288C
MBS50-1	*MATa*, *SUL*^{R}	3.6	UV light-induced mutant from BD-4a
MBS50-2	*MATa*, *SUL*^{R}	3.6	UV light-induced mutant from BD-4a
MBS50-6	*MATa*, *SUL*^{R}	3.6	UV light-induced mutant from BD-4a

[a] M.I.C., Minimal Inhibitory Concentration
[b] *sul*^{S} and *SUL*^{R}, sensitivity or resistance to sulphite

medium containing 1% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose and adjusted to pH 3.85 by using a sodium citrate buffer to reach a 30 mM final concentration. Sulphite was included in media by incorporating portions of a freshly prepared solution of concentrated sodium sulphite to give the desired final concentration. Starter cultures, 20 ml of YEPD in 100 ml flasks, were inoculated with a single fresh yeast colony. After 12 h on an orbital shaker at 28 °C the cultures were diluted in 300 ml of YEPD to give, if not otherwise specified, a final concentration of 0.05 mg dry weight ml^{-1} and incubated at 28 °C as reported above. Yeast growth was monitored by measuring the optical density at 600 nm, which was related to yeast mg dry weight by a standard curve.

Genetic analysis. Matings were performed as described by Sherman et al. (1986). Diploid clones were purified and tested for their ability to sporulate. Sporulation and tetrad analysis were performed as described by Mortimer and Hawthorne (1969).

Cell-free extract preparation. Yeast cells harvested at 1 mg dry weight ml^{-1} were disrupted as previously reported (Casalone et al. 1988). Crude extracts were used to determine GSH concentration, glyceraldehyde-3-phosphate dehydrogenase activity, and protein concentration (Lowry et al. 1951).

Glutathione determination. GSH was determined as reported by Akerboom and Sies (1981) with some modifications (Casalone et al. 1988).

Glyceraldehyde-3-phosphate dehydrogenase (G3PD) inactivation by sulphite. Enzyme inactivation has been studied both in whole cells and in cell-free extracts. In the first case cells were incubated with 1 mM or 2 mM of sulphite, samples were taken at different times and G3PD activity was assayed on crude extracts after cell disruption. In the second case, cell-free extracts from cells grown in a sulphite-free medium were incubated with 1 mM or 2 mM of sulphite at 37 °C and aliquots of the incubation mixture were assayed for G3PD activity at different times. G3PD activity was assayed as reported by Beutler (1975).

^{35}S-sulphite uptake. To measure the extent of sulphite accumulation in yeast cells, we followed essentially the method reported by Pilkington and Rose (1988). Yeast cultures grown in YEPD up to 1.0–1.2 mg dry weight ml^{-1} were washed twice with 30 mM of sodium citrate buffer (pH 3.0) containing 100 mM of glucose, suspended in the same buffer at 10 mg dry weight ml^{-1} and allowed to equilibrate for 10 min at 30 °C. A portion of this cell suspension was added to a reaction mixture containing 30 mM of citrate buffer (pH 3.0), 100 mM of glucose and 0.1 mM of ^{35}S-sulphite (1.0 μCi ml^{-1}) pre-equilibrated at 30 °C, to give a final cell concentration of about 1.5 mg dry weight ml^{-1}. This suspension was incubated at 30 °C with gentle shaking and, at appropriate time intervals, 0.5 ml samples were filtered through 0.45 μm pore size, 25 mm diameter, filters (Millipore), which were then quickly washed with 5 ml of buffered 0.1 mM sulphite solution. Finally, 0.2 ml of Soluene (Packard) and, after 10–15 min, 7 ml of Hionic Fluor (Packard) were added to the filters in scintillation vials. Radioactivity was measured in a Kontron BETAmatic II liquid scintillation counter (Heraeus). Background activity was estimated by repeating the procedure with cell-free medium to check washing efficiency and to quantify any sulphite retained on the filters.

Analytical methods. Fifty milliliter aliquots of YEPD in 250 ml flasks were inoculated at a density of 0.5 mg dry wt ml^{-1} with starter cultures. At different time intervals, OD 600 nm was determined for each culture, an aliquot was centrifuged and the cell-free medium assayed for acetaldehyde and pyruvate by using kits from Boehringer. Acid-soluble thiols were measured following the method of Ellman (1959).

Results

Genetic analysis of sulphite-resistant mutants

In order to analyze dominance and segregation of sulphite resistance, seven independent sulphite-resistant (Sul^{R}) mutants derived from the two different *S. cerevisiae* strains, S288C and BD-4a, were crossed to sensitive strains of opposite mating type. All the purified, heterozygous diploid clones derived from each cross showed a Sul^{R} phenotype, indicating that resistance was due to dominant mutations (data not shown). Furthermore, sulphite resistance segregated as a single-gene mutation in all the examined heterozygous diploids. In fact, random analysis of about 90 spores from each independent diploid showed a 1:1 segregation of resistant and sensitive spores (data not shown), and tetrad analysis performed on one of these heterozygous strains (MBS20-9 by 1425) confirmed a 2:2 segregation of resistant and sensitive spores in each of the 18 asci analysed (Table 2).

To verify whether the seven independent dominant *SUL*^{R} mutations identified the same gene, mutants were crossed to each other and random spore analysis was performed on about 90 meiotic products from each cross to see whether any Sul^{S} segregant would originate from some of the crosses. As shown in Table 2, diploids carrying all the necessary pairwise combinations of mutations could only segregate Sul^{R} spores, as expected if all mutations fall in the same locus. For three out of seven crosses listed in Table 1 these results were confirmed by tetrad

Table 2. Allelism test on independent sulphite-resistant mutants

Crosses	Random spore analysis		Tetrad analysis[b]	
	No. of analysed spores	No. of Sul^R spores	No. of analysed tetrads	No. of tetrads with 4:0 segregation for Sul^R:Sul^S
MBS50-1 × MBS20-10	85	85	ND	ND
MBS50-2 × MBS20-10	83	83	ND	ND
MBS50-6 × MBS20-10	85	85	23	23
MBS3-57-3 × MBS20-10[a]	89	89	24	24
MBS3-57-3 × MBS20-7	89	89	ND	ND
MBS3-57-3 × MBS20-12	65	65	ND	ND
MBS3-57-3 × MBS30	85	85	23	23
MBS20-9 × 1425[c]	86	46	18	0

[a] MBS3-57-3 (*MATa, ade1, SUL*^{R}) is a haploid segregant from the cross of the diploid obtained by crossing MBS20-9 *MATα, SUL*^{R} by 1425 *MATa, ade1 his1, sul*^{S}

[b] ND, not determined

[c] All the analyzed tetrads from the control cross showed a 2 Sul^R:2 Sul^S spore segregation, as expected

Table 3. Intracellular glutathione and protein content of *S. cerevisiae* S288C and its sulphite-resistant derivatives MBS20-9 and MBS30 after different times of incubations in the presence of 1 mM of sulphite. The data reported are the average of three experiments plus or minus the standard error

Strain	Time of incubation (min)	Glutathione nmol (mg dry wt)$^{-1}$	Protein μg (mg dry wt)$^{-1}$
S288C	0	6.3 (±1.0)	222 (±60)
	45	4.6 (±1.1)	190 (±18)
	90	5.1 (±0.6)	221 (±33)
MBS20-9	0	8.3 (±2.3)	262 (±19)
	45	6.2 (±1.4)	227 (±36)
	90	6.9 (±1.9)	249 (±22)
MBS30	0	8.0 (±2.1)	243 (±57)
	45	5.6 (±1.1)	220 (±53)
	90	5.7 (±0.7)	235 (±48)

analysis. Taken together, these data suggest that a major gene is responsible for sulphite resistance in *S. cerevisiae*.

Effect of sulphite on protein and glutathione cell content

The effect of 1 mM of sulphite on the GSH levels of yeasts was assessed by adding it to mid exponential-phase cultures, and by measuring the changes in protein and GSH content of the cells over the following 90 min.

The glutathione and protein content of the strains did not change significantly (Table 3).

Glyceraldehyde-3-phosphate dehydrogenase inactivation by sulphite

The effect of sulphite on the G3PD activity in whole cells and in cell-free extracts of the S288C, MBS20-9 and MBS30 strains was assessed as reported in Materials and methods.

Table 4. Time-course of inactivation of glyceraldehyde-3-phosphate dehydrogenase after incubation with sulphite of whole cells of strains S288C, MBS20-9 and MBS30

Strain	Time of incubation (min)	Activity (%)[a]	
		1 mM sulphite	2 mM sulphite
S288C	0	100	100
	45	33	0
	90	138	0
MBS20-9	0	100	100
	45	118	91
	90	153	82
MBS30	0	100	100
	45	125	95
	90	105	85

[a] G3PD activities are reported as per cent of the time-zero values, which were 1.33, 1.88 and 1.54 μmol (min mg protein)$^{-1}$ for the parental, MBS20-9 and MBS30 strains respectively. The data are the means of three experiments, each carried out in duplicate [the mean SEs of the estimates were 0.50 and 0.54 for 1 mM and 2 mM of sulphite, respectively, calculated with the values expressed as μmol (min mg protein)$^{-1}$]

Inactivation of G3PD during the incubation of whole cells with sulphite, reported in Table 4, is expressed as the per cent residual activities of the zero-time values. During incubation with 1 mM of sulphite, the G3PD activity of the parental strain was reduced to about one-third by 45 min, but it was fully restored 45 min later. With 2 mM of sulphite, enzyme activity was already completely inhibited in the parental strain after 45 min of incubation, while it was only slightly affected in the mutants even after 90 min.

G3PD inactivation in cell-free extracts with 1 mM of sulphite is reported in Fig. 1. Both parental and mutant strains showed a 50% inactivation of G3PD at 45 min of incubation. No G3PD activity was detected in extracts of all the strains after a 30 min incubation with 2 mM of sulphite (data not shown).

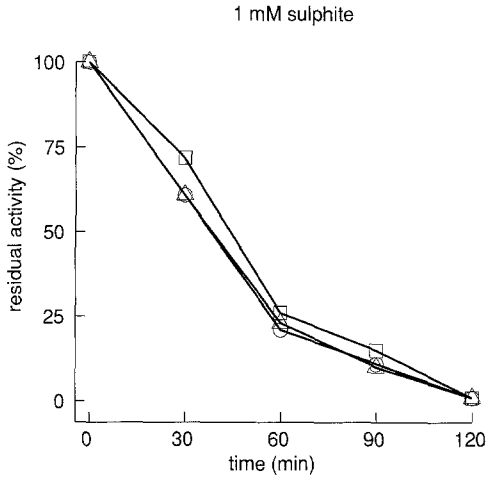

Fig. 1. Time-course of inactivation of glyceraldehyde 3-phosphate dehydrogenase in cell-free extracts of *S. cerevisiae* S288C (○), MBS20-9 (△) and MBS30 (□) by 1 mM of sulphite. G3PD activities are expressed as the percentage of the time-zero values, which were 0.96, 1.36 and 1.15 μmol (min mg protein)$^{-1}$ for S288C, MBS20-9 and MBS30, respectively. The mean SE of the estimates of three different experiments, each carried out in duplicate, is 0.16

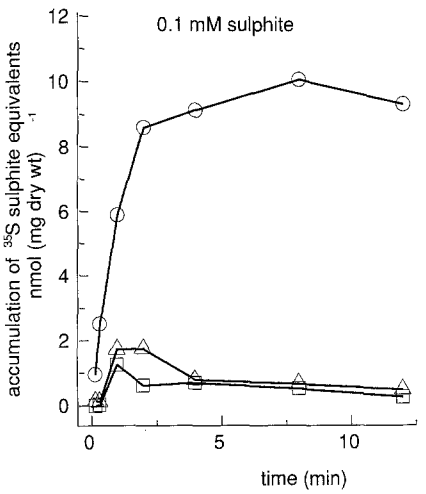

Fig. 2. Time-course of accumulation of ^{35}S-sulphite equivalents in *S. cerevisiae* S288C (○), MBS20-9 (△) and MBS30 (□) at 30 °C, 1.0 mg dry weight cells ml^{-1} in 30 mM of citrate buffer (pH 3.0) containing 100 mM of glucose in the presence of 0.1 mM of ^{35}S-sulphite (0.1 μCi ml^{-1}). The values reported are the means from four different experiments, each carried out in duplicate. Mean SE of the estimates is 1.02

^{35}S-sulphite uptake

For ^{35}S-sulphite experiments cells were suspended in an aqueous solution buffered at pH 3.0 and containing 100 mM of glucose.

The data, reported in Fig. 2, showed that with 0.1 mM of sulphite MBS20-9 and MBS30 sulphite-resistant mutants differed greatly from the parental strain S288C in their sulphite accumulation pattern. Wild-type cells accumulated up to 10 nmol of ^{35}S-sulphite equivalents per mg dry weight, whereas MBS-20.9 and MBS-30 accumulated only 1.75 and 1.26 nmol per mg dry weight, respectively.

Extracellular production of sulphite-binding compounds

Sulphite was added to 50 ml of mid-exponential phase cultures, and cell density, as well as the excretion of acetaldehyde, pyruvate and acid soluble thiols in the medium, was monitored at various time intervals.

Growth of the parental strain S288C was inhibited by the addition to the culture medium of 0.6 mM of sulphite, a concentration that did not affect the growth of the MBS20-9 and MBS30 mutants (Fig. 3a). No changes in the pH of the medium during the 9 h of incubation, with or without sulphite, were observed for any of the three strains (data not shown).

Acetaldehyde production was followed in yeast cultures grown both in the presence and the absence of sulphite (Fig. 3b). In the absence of sulphite both mutants showed a higher acetaldehyde accumulation compared to the parental strain, the levels of acetaldehyde in the medium increasing from practically zero to 2.5 mM for the mutants and to 0.8 mM for the parental strain after 9 h of growth. For all three strains, sulphite addition did not seem to affect acetaldehyde accumulation in the medium.

No significant changes in pyruvate and acid-soluble thiol production, with or without sulphite, were observed for any of the three strains (data not shown).

Discussion

The physiological and biochemical basis of sulphite resistance in yeasts has been investigated by comparative analysis of yeast species which differed in their sensitivity to sulphite (Stratford et al. 1987; Pilkington and Rose 1988, 1989). In these studies, it has been suggested that variations in the production and excretion of sulphite-binding compounds (particularly acetaldehyde) in the lipid composition of the plasma membrane and in the intracellular buffering capacity might be responsible for a different sensitivity to sulphite.

We had previously reported the isolation of spontaneous and UV-induced sulphite-resistant *S. cerevisiae* mutants and their preliminary characterization (Casalone et al. 1989). In the present work we have further characterized two (MBS20-9 and MBS30) out of seven mutants, which appear to carry dominant mutations in the same single gene. Similar results have been reported by Bakalinsky and Snow (1990). The work of Guerra et al. (1981) and Thorton (1982) suggests that two genes or multiple dominant genes, respectively, are involved in sulphite tolerance.

Hinze and Holzer (1986) have attributed to the sulphite-dependent inactivation of G3PD the rapid and deleterious decrease in the content of ATP following exposure of yeast cells to sulphite (Schimze and Holzer 1979). Our data indicate that sulphite inactivation of G3PD in cell-free extracts was very similar in parental and mutant strains, suggesting that resistance cannot be

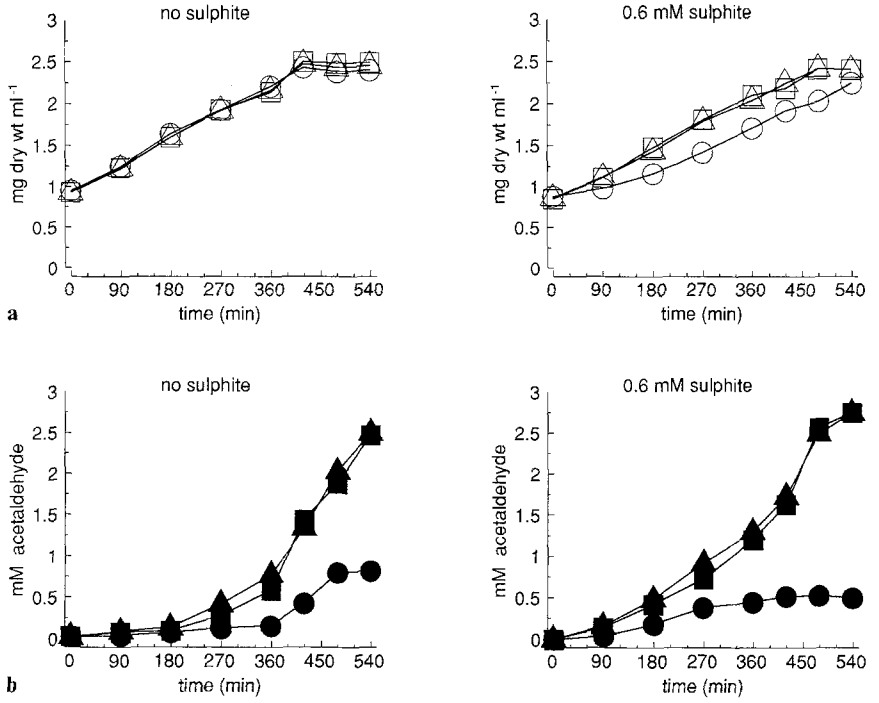

Fig. 3a, b. The effect of the addition of sulphite on the growth **a** and the excretion **b** of acetaldehyde in the S288C (○, ●), MBS20-9 (△, ▲) and MBS30 (□, ■) strains of *S. cerevisiae*. The average values from five and three different experiments for zero and 0.6 mM of sulphite, respectively, are reported. Average SEs of the estimates are 0.08 and 0.09 **a**, and 0.16 and 0.24 **b** with no sulphite and with 0.6 mM of sulphite, respectively

explained by changes in the sulphite sensitivity of this enzyme. The lower inhibition of G3PD activity observed in the mutants when whole cells were incubated with sulphite might be explained by the lower sulphite-uptake of the resistant cells.

Since a positive correlation between high intracellular concentration of GSH and sulphite tolerance has been found (Kagedal et al. 1986; Grill et al. 1982; Chiment et al. 1986; Scardovi 1952; Casalone et al. 1989) in a number of organisms, we have measured the GSH content in mutants and wild-type strains in the presence of sulphite. In our hands, sulphite addition did not significantly modify the intracellular concentration of GSH in MBS20-9, MBS30 and S288C cells.

Permeability to sulphite appeared to be modified in the two resistant mutants. ^{35}S-sulphite uptake experiments showed that the acquisition of resistance to sulphite in *S. cerevisiae* was correlated with an enhanced ability to exclude sulphite since sulphite accumulation was much reduced in MBS20-9 and MBS30 compared to the parental strain S288C.

Acetaldehyde production has also been invoked as a factor involved in the de-toxification of sulphite in yeasts (Stratford et al. 1987). Both excretion of acetaldehyde in cultures of *S. cerevisiae* supplemented with sulphite and the ability of this carbonyl compound to bind sulphite to form α-hydroxysulphonate (Burroughs and Sparks 1964) are well known phenomena.

We found that the sulphite-resistant mutants MBS20-9 and MBS30 accumulated much more acetaldehyde in the medium than the parental strain, both in the presence and the absence of sulphite. These data corroborate the hypothesis that acetaldehyde production could be involved in sulphite resistance (Stratford et al. 1987).

At present we cannot establish the relative roles of the intracellular accumulation of sulphite and the extracellular accumulation of acetaldehyde in the mechanism of resistance. Cloning of the gene involved in sulphite resistance, which is in progress, could provide additional information about the mechanism of resistance, which is interesting both from the basic and applied points of view. In fact, sulphites are added at the beginning of the wine-making process to inhibit bacteria and wild yeast growth, but sulphite addition may also delay the growth of the wine yeast *S. cerevisiae*; therefore the use of sulphite-resistant strains could reduce this undesirable effect.

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