Sulphur Dioxide Resistance in *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*

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Saccharomyces cerevisiae was unable to grow in media containing above about 1.5 mM free sulphite at pH 4.0, whereas *Saccharomycodes ludwigii* grew at the same pH value in the presence of 7.8 mM free sulphite. Expressed in terms of μl of intracellular water, the initial velocity of sulphite accumulation by *S. codes ludwigii* was approximately twice that of *S. cerevisiae*, although the former yeast accumulated at equilibrium only about one-third of the amount of sulphite accumulated by *S. cerevisiae*. A Woolf–Hofstee plot for accumulation of SO_2 by *S. codes ludwigii* at pH 3.0 and 30 °C gave a vertical line. Incorporation of sulphite in growth media induced excretion of acetaldehyde by both yeasts, the rate being greater by *S. codes ludwigii* than *S. cerevisiae*. Acetaldehyde excretion was accompanied by release of lower concentrations of pyruvate. Excretion of 2-oxoglutarate was barely detectable. It is suggested that the greater resistance of *S. codes ludwigii* to sulphite, compared with *S. cerevisiae*, may be explained partly by its decreased capacity to accumulate the compound, and partly by its ability to produce more acetaldehyde.

INTRODUCTION

Sulphite has long been known to be a powerful antimicrobial compound (Hammond & Carr, 1976), although certain species of yeast are comparatively resistant to its action. Two factors complicate any study of the reaction of micro-organisms to sulphite. Firstly, the compound exists in three forms depending on the pH value. At pH values below 1.8, sulphite exists predominately as free SO_2 , and at pH values above 7.2 largely as SO_3^{2-} ; at intermediate pH values, it exists in various proportions as the bisulphite ion (HSO_3^-). The pK value for the equilibrium $\text{SO}_2 \rightleftharpoons \text{HSO}_3^-$ is 1.77, and for the equilibrium $\text{HSO}_3^- \rightleftharpoons \text{SO}_3^{2-}$ 7.20 (King *et al.*, 1981). In this paper, the term 'sulphite' is used to denote collectively all three forms. Secondly, sulphite reacts with compounds containing carbonyl groups to form α -hydroxysulphonates, usually referred to as 'bound sulphite' (Burroughs & Sparks, 1964a).

Although strains of *Saccharomyces cerevisiae* are among the more sulphite-resistant of micro-organisms (Wedzicha, 1984), little is known of the physiological basis of this resistance. At least some resistance is attributable to production of sulphite-binding compounds, primarily acetaldehyde, 2-oxoglutarate and pyruvate, when organisms are grown in the presence of sulphite (Rankine, 1968; Rankine & Pocock, 1969; Weeks, 1969). It is also conceivable that differences in sulphite resistance of yeasts may be caused, in part at least, by different rates of transport of sulphite. Of the three molecular forms of sulphite, only free SO_2 is transported into *S. cerevisiae* (Macris & Markakis, 1974). Stratford & Rose (1986) showed that SO_2 transport into *S. cerevisiae* is by free diffusion, so raising the possibility that the lipid composition of the plasma membrane may affect sulphite resistance in this yeast. Another yeast, *Saccharomycodes ludwigii*,

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is generally reckoned to be even more resistant to sulphite than *S. cerevisiae*, and is often isolated from alcoholic beverages that have been treated with sulphite at concentrations which prevent growth of *S. cerevisiae* (Amerine & Kunkee, 1968; Beech & Carr, 1977). Nothing has been reported on the physiological basis of sulphite resistance in *S. codes ludwigii*. The present paper compares the physiology of the reaction to sulphite of strains of *S. cerevisiae* and *S. codes ludwigii*.

METHODS

Organisms. The yeasts used, *S. cerevisiae* strain TC8 (Stratford and Rose, 1985), and *S. codes ludwigii* TC10, both provided by T. C. Cowland of the Taunton Cider Co, Norton Fitzwarren, Somerset, UK, were maintained on slopes of malt extract-yeast extract-glucose-mycological peptone (MYGP) agar (Wickerham, 1951).

Experimental cultures. The medium used was a modification of that described by Alterthum & Rose (1973) and contained (l⁻¹): glucose, 20 g; (NH₄)₂SO₄, 3.0 g; KH₂PO₄, 3.0 g; yeast extract (Difco), 1.0 g; MgSO₄·7H₂O, 25 mg; and CaCl₂·2H₂O, 25 mg. The medium was buffered to pH 4.0 by addition of 5.7 g citric acid and 6.0 g trisodium citrate l⁻¹. Sulphite was included in media by incorporating portions of a freshly prepared (Postgate, 1963) solution of 78 mM-sodium metabisulphite to give the desired sulphur dioxide concentration. Portions (1 l) of sterile medium in 2 l round flat-bottomed flasks were inoculated with a starter culture to give experimental cultures containing 20 mg dry wt organisms l⁻¹. Starter cultures were prepared by inoculating 100 ml portions of medium in a 250 ml conical flask with a pinhead of yeast from a slope culture, and incubating for approximately 24 h on an orbital shaker (300 r.p.m.) at 30 °C. Experimental cultures were incubated at 30 °C with stirring by a magnetic stirrer bar (50 mm length). Growth was followed by measuring the optical density of portions of cultures at 600 nm; these measurements were converted to mg dry wt organisms ml⁻¹ using a calibration curve for each yeast. Organisms were harvested from late exponential-phase cultures (0.5 mg dry wt ml⁻¹) by centrifugation for 1 min at 5000 g and 4 °C, and washed twice with 10 ml 30 mM-citrate buffer (pH 4.0 unless otherwise stated). Alternatively, they were harvested by filtration through a membrane filter (0.45 µm pore size; 50 mm diam.; Oxoid) and washed twice with 10 ml citrate buffer.

Viability measurements. Portions of culture were serially diluted in one-quarter strength Ringer's solution, and appropriate dilutions plated in duplicate on MYGP agar plates which were incubated for 3 days at 30 °C.

Disruption of organisms. Organisms were harvested by centrifugation to obtain a pellet containing 50 mg dry wt equivalent. The pellet was resuspended in a minimal amount of sterile ice-cold citrate buffer (30 mM) and added to 30 g glass beads (0.17–0.18 mm diam.; Braun) with sufficient buffer to form a thick slurry. The organisms were disrupted in a Braun homogenizer, cooled by liquid CO₂, for a total period of 2 min. Large cellular debris was removed by centrifugation for 10 min at 1200 g and 4 °C. The supernatant was retained and stored at 4 °C.

Measurement of initial velocities of SO₂ accumulation. Organisms were harvested from late exponential-phase cultures (0.5 mg dry wt ml⁻¹) by filtration, washed twice with 10 ml 30 mM-citrate buffer containing 100 mM-glucose, resuspended in 5 ml of the same buffer to 3.0–6.0 mg dry wt ml⁻¹ and equilibrated at 30 °C for 3 min. A portion (1 ml) of the suspension was drawn into a BCL 8000 repetitive pipette (Boehringer) fitted with a 6 ml syringe (Boehringer) followed immediately by 5 ml citrate buffer containing 100 mM-glucose, 1.0 µM – 5.0 mM-sulphite and [³⁵S]sulphite (1.85–0.74 kBq ml⁻¹). Portions (0.5 ml) of the suspension were rapidly filtered at 1 or 2 s intervals through membrane filters (0.45 µm pore size; 25 mm diam; Millipore) and the filters with organisms quickly washed with 2 ml citrate buffer containing 100 mM-glucose and non-radioactive sulphite at the concentration used in the experiment. Filters were prewashed with 10 ml 10 mM-non-radioactive sulphite (pH 4.0). Heat loss from the suspension over a 10 s sampling period was negligible. Filters with organisms were placed in scintillation vials containing 7 ml Optiphase Safe (Fisons). Radioactivity in the vials was measured in a LKB RackBeta liquid scintillation spectrometer (model 1217). The suspension remaining in the pipette at the end of a sampling period was ejected into a scintillation vial containing Optiphase to check on the total radioactivity of the suspension. Accumulation of sulphite over longer periods (up to 5 min) was followed using stirred suspensions of organisms in citrate buffer supplemented with 100 mM-glucose and sulphite containing [³⁵S]sulphite as described by Stratford & Rose (1986).

Relationship between dry wt of organisms and cytoplasmic volume. This was established by the method of Cole & Keenan (1987). Organisms were suspended in a solution containing ³H₂O, which equilibrates with both extracellular and intracellular water, and [¹⁴C]mannitol which equilibrates with interstitial water and water in the wall but does not penetrate the plasma membrane. The difference in the extent of dilution of each labelled compound after mixing was used to calculate the relationship between dry wt of organisms and cytoplasmic volume. Portions (1.0 ml) of a thick suspension (40 mg dry wt ml⁻¹) were transferred to Eppendorf tubes, to pairs of which was added 0.1 ml of a solution containing either ³H₂O (60 MBq mol⁻¹; final concn 61.6 µM) or D-[¹⁴C]mannitol (2.02 MBq µmol⁻¹; final concn 1.8 nM). The contents of the tubes were then mixed by inversion and left to stand at room temperature (20–22 °C) for 5 min. After centrifuging the tubes in a MSE Microcentaur (12000 g, 20 s, 20 °C), portions (0.1 ml) of the supernatant were removed from each tube and added to scintillation

vials containing 2 ml Unisolve 1 (Koch-Light). Radioactivity in the vials was measured in a LKB RackBeta liquid scintillation spectrometer. *S. cerevisiae* contained 2.61 μl and *S'codes ludwigii* 1.43 μl (mg dry wt) $^{-1}$.

Analytical methods. Total SO_2 was measured by the method of Burroughs & Sparks (1963) and free SO_2 by the Burroughs & Sparks (1964b) method. Acetaldehyde was determined by a modification of the Burroughs & Sparks (1961) method. Culture filtrate (20 ml) was placed in a Markham steam-distillation apparatus, supplemented with 5 ml phosphate buffer (35 mM; pH 7.0) and sufficient 1 M-NaOH to neutralize the sample. The sample was then steam distilled for about 5 min into 5 ml buffered $\text{Na}_2\text{S}_2\text{O}_3$ (56 g l^{-1} ; pH 7.0) containing Na_2EDTA (5 g l^{-1}). To titrate the excess uncomplexed sulphite, the distillate was supplemented with 1 ml each of 25% (v/v) HCl and 1% (w/v) freshly prepared starch solution, and then titrated to a faint blue-black colour with iodine solution (0.1 M followed by 0.01 M). Solid NaHCO_3 was added to neutralize the HCl, thereby dissociating the acetaldehyde-sulphite complex and liberating sulphite in the distillate, which was immediately titrated to a blue-black endpoint with 0.01 M-iodine solution. The latter titre volume multiplied by 11.0 (L. F. Burroughs, personal communication) gave the acetaldehyde concentration ($\mu\text{g l}^{-1}$). Pyruvate was determined using a test kit (Boehringer) according to the method of Czok & Lamprecht (1974), and 2-oxoglutarate as described by Bergmeyer & Bernt (1974).

Chemicals. All reagents used were AnalaR or of the highest grade available commercially. Amersham supplied $^3\text{H}_2\text{O}$, [^{14}C]mannitol and sodium [^{35}S]sulphite which was stored at -20°C in 5 mM-EDTA to avoid oxidation.

RESULTS

Effect of sulphite on growth and viability

Incorporating sulphite in media caused an increase in the lag phase of growth of both yeasts (Fig. 1). *S. cerevisiae* was unable to grow in the presence of 1.56 mM-sulphite at pH 4.0, but *S'codes ludwigii* was able to grow at this pH value in the presence of higher concentrations of sulphite (Fig. 1) up to and including 7.8 mM (not shown) although the lag phase at this concentration was about 41 h. *S. cerevisiae* was also much more sensitive than *S'codes ludwigii* to the lethal effect of sulphite, as assessed by the decline in viability of organisms suspended in media containing different concentrations of sulphite (Fig. 2).

Velocities and extent of sulphite accumulation

The initial velocity of sulphite accumulation (1.0 mM, pH 4.0) by *S. cerevisiae* was 47.5 ± 4.9 nmol (mg dry wt) $^{-1}$ min $^{-1}$, while that by *S'codes ludwigii* was 53.9 ± 7.0 nmol (mg dry wt) $^{-1}$ min $^{-1}$. These values are means of at least six separate determinations. Calculated on the basis of μl of intracellular water, the initial velocity of accumulation into *S. cerevisiae* was

Fig. 1. Time-course of growth of *S. cerevisiae* (a) and *S'codes ludwigii* (b) in media (pH 4.0) either lacking sulphite (○) or containing 0.31 mM (●), 0.78 mM (□), 1.56 mM (■) or 3.12 mM (△) total sulphite. Initial free sulphite concentrations were, respectively, 0.0, 0.20, 0.66, 1.43 and 2.95 mM.

Fig. 2. Time-course of change in viability of populations of *S. cerevisiae* (a) and *S. codes ludwigii* (b) incubated with stirring at 30 °C in media either lacking sulphite (○), or containing 0.31 mM (●), 0.78 mM (□), 1.56 mM (■), 7.80 mM (△) or 15.60 mM (▲) total sulphite. Values given are the means of duplicate determinations.

Fig. 3

Fig. 3. Time-course of accumulation at 30 °C of sulphite equivalents by *S. cerevisiae* (○) and *S. codes ludwigii* (●), each at 1.0 mg dry wt ml⁻¹, from 30 mM-citrate buffer (pH 3.0) containing glucose (100 mM) and [³⁵S]sulphite (0.1 mM). Values quoted are means of at least six separate determinations.

Fig. 4

Fig. 4. Woolf-Hofstee plot for accumulation of molecular SO₂ by *S. codes ludwigii* suspended in buffer at pH 3.0 and 30 °C. Concentrations of molecular SO₂ were calculated from the data of King *et al.* (1981). Bars indicate SD.

18.2 nmol μl⁻¹ min⁻¹, and into *S. codes ludwigii* 37.7 nmol μl⁻¹ min⁻¹. Under the same conditions, net accumulation by *S. cerevisiae* ceased at a value of 50.0 ± 1.8 nmol (mg dry wt)⁻¹ and by *S. codes ludwigii* at 8.8 ± 1.7 nmol (mg dry wt)⁻¹ (Fig. 3). In terms of μl of intracellular water, net accumulation values were 19.1 nmol μl⁻¹ for *S. cerevisiae* and 6.2 nmol μl⁻¹ for *S. codes ludwigii*. A Woolf-Hofstee plot (Hofstee, 1959) for accumulation of SO₂ by *S. codes ludwigii* at pH 3.0 and 30 °C gave a vertical line (Fig. 4).

Sulphite-induced production of binding compounds

Growth of *S. cerevisiae* and *S. codes ludwigii* in media supplemented with sulphite was accompanied, over the first few hours of incubation, by disappearance of free sulphite from the

Fig. 5. Time-course of decline in the concentration of free sulphite (○, □) and of the increase in acetaldehyde concentration (●, ■) in filtrates from cultures of *S. cerevisiae* (a) and *S. codes ludwigii* (b) during the early stages of growth.

medium and excretion of acetaldehyde. In media lacking SO_2 , neither yeast produced acetaldehyde. However, in the presence of SO_2 the rate of production of acetaldehyde was greater by *S. codes ludwigii* than by *S. cerevisiae*. In a medium containing initially 0.55–0.60 mM free SO_2 , *S. cerevisiae* produced acetaldehyde at a rate of $88 \mu\text{mol l}^{-1} \text{h}^{-1}$, whereas *S. codes ludwigii* produced it at a rate of $126 \mu\text{mol l}^{-1} \text{h}^{-1}$ (Fig. 5). During suspension of *S. cerevisiae* in media supplemented with sulphite in the range 0.20–0.72 mmol l^{-1} , acetaldehyde was produced in amounts that represented a mean excess of about 4% over those required to bind the sulphite initially present. *S. codes ludwigii*, suspended in media with initial sulphite concentrations in the range 0.55–5.83 mmol l^{-1} , produced acetaldehyde in amounts that were on average 25% in excess of those required to bind the sulphite initially present. Sulphite-induced production of acetaldehyde was accompanied by production of pyruvate in amounts that, in cultures of *S. cerevisiae*, were 20–40% of the amount of acetaldehyde produced; in cultures of *S. codes ludwigii* they were negligible. Production of 2-oxoglutarate was barely detectable in any of the cultures. When supernatants (10 ml; pH 4.0) from suspensions of 50 mg dry wt equivalent of disrupted *S. cerevisiae* or *S. codes ludwigii* were supplemented with sulphite (92.8 mM) and incubated at 4 °C for 30 min, virtually identical proportions of sulphite were bound in both mixtures.

DISCUSSION

Although it has often been claimed that *S. codes ludwigii* is more resistant to sulphite than *S. cerevisiae*, which itself is more sulphite-resistant than most other yeasts (Hammond & Carr, 1976; Beech & Carr, 1977), this paper is the first to describe differences in sulphite sensitivity between these two yeasts in quantitative terms. In sulphite-containing media, both yeasts showed an increase in the lag phase of growth and a loss of colony-forming ability. While these effects were observed with *S. cerevisiae* in the range of sulphite concentrations in which they were examined by Schimz (1980), they were only observed to take place with *S. codes ludwigii* at very much higher concentrations. Transport of SO_2 into *S. cerevisiae* at pH 3.0 is by simple diffusion (Stratford & Rose, 1986). The vertical Woolf–Hofstee plot obtained in the present study with *S. codes ludwigii*, also examined at pH 3.0 to increase the concentration of SO_2 , indicates that this yeast does not have a finite Michaelis constant for molecular SO_2 , which in turn suggests that passage of SO_2 into it is also by simple diffusion.

A significant finding made in the present study was that the amount of sulphite accumulated intracellularly was greater in *S. cerevisiae* than in *S. codes ludwigii*. Expressed in the physiologically more significant terms of μl cytoplasmic volume, initial velocities of sulphite accumulation were greater with *S. codes ludwigii* than with *S. cerevisiae*. This finding is of interest in relation to the report (Kaneko *et al.*, 1976) that *S. codes ludwigii* is richer than *S. cerevisiae* in $\text{C}_{18:1}$ phospholipid fatty-acyl residues which might indicate that a greater membrane fluidity facilitates diffusion of SO_2 across the plasma membrane.

Although sulphite, mainly in the form of molecular SO_2 , has been shown to react with a wide variety of cell constituents, principally molecules containing carbonyl groups (Wedzicha, 1984), it is now thought that the initial reaction of *S. cerevisiae* with sulphite, at concentrations up to 10–15 mM, leads to depletion of cellular ATP (Schimz & Holzer, 1979) mainly as a result of inactivation of glyceraldehyde-3-phosphate dehydrogenase (Hinze & Holzer, 1986). It is very likely that *S. cerevisiae* and *S. codes ludwigii* contain ATP pools of approximately the same size, and also produce ATP at about the same rates. To explain the different reaction of these yeasts to sulphite, one possibility is that they differ in the extent to which exogenous molecular SO_2 has access to those intracellular targets that are involved in ATP generation. An alternative explanation is that glyceraldehyde-3-phosphate dehydrogenases in the two yeasts differ in sensitivity to SO_2 .

When molecular SO_2 enters the yeast cell by diffusion, it encounters an environment which is at a higher pH value than that prevailing outside the cell. As a result, a proportion of the SO_2 entering the cell is converted into HSO_3^- which, since it cannot leave the cell, forms an effective intracellular sulphite trap (Stratford & Rose, 1986), the magnitude of which will depend on the intracellular pH value. Another possible explanation, therefore, for the differences observed in the present study in the reaction of *S. cerevisiae* and *S. codes ludwigii* to sulphite is that, assuming equal internal buffering capacity, the latter yeast has a lower internal pH value than *S. cerevisiae*.

Another factor which must be taken into account in any explanation of the different responses of the two yeasts to sulphite is the greater production of acetaldehyde by *S. codes ludwigii*. However, this is unlikely to help explain the smaller uptake of sulphite by this yeast, since data on net accumulation were obtained using organisms suspended in solutions lacking a carbon source and so under conditions that would not induce organisms to produce significant amounts of acetaldehyde. Nor is there evidence using extracts of organisms of a difference in the concentration of sulphite-binding compounds inside the two yeasts. The relevance of the different capacities to produce acetaldehyde in the presence of sulphite, in relation to their different sulphite tolerances, remains to be elucidated.

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