Reactions of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* to Sulphite

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Sulphite inhibited growth of all four yeasts studied, *Zygosaccharomyces bailii* NCYC 563 being most sensitive and *Saccharomyces cerevisiae* NCYC 431 the least. Vertical Woolf–Eadie plots were obtained for initial velocities of ^{35}S accumulation by all four yeasts suspended in high concentrations of sulphite. Equilibrium levels of ^{35}S accumulation were reached somewhat faster with strains of *S. cerevisiae* than with those of *Z. bailii*. With all four yeasts, the greater the extent of ^{35}S accumulation, the larger was the decline in internal pH value. Growth of *S. cerevisiae* TC8 and *Z. bailii* NCYC 563, but to a lesser extent of *S. cerevisiae* NCYC 431 and *Z. bailii* NCYC 1427, was inhibited when mid exponential-phase cultures were supplemented with 1.0 or 2.0 mM-sulphite, the decrease in growth being accompanied by a decline in ethanol production. Unless growth was completely inhibited, the sulphite-induced decline in growth was accompanied by production of acetaldehyde and additional glycerol.

INTRODUCTION

Sulphite has long been recognized as a powerful antimicrobial agent (Hammond & Carr, 1976). The compound exists in solution in three forms, the proportions of which depend on pH value. At pH values below 1.8, sulphite exists predominantly 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^- ; King *et al.*, 1981). The antimicrobial action of sulphite is greatest at low pH values (Wedzicha, 1984), which explains why the compound is particularly effective against yeasts which, in general, grow best at pH values in the range 3.0–5.0 (Rose, 1987). The greater antimicrobial action of sulphite against *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii* at low pH values has been explained by the discovery that, of the three molecular forms in which sulphite exists in solution, only SO_2 enters these organisms (Stratford & Rose, 1986; Stratford *et al.*, 1987). Yeast species differ considerably in their ability to resist the antimicrobial action of sulphite. Warth (1985) found that *Kloeckera apiculata* and *Hansenula anomala* were much more sensitive to sulphite than strains of *S. cerevisiae* which is generally recognized as being a sulphite-resistant yeast. A yeast which has been reported to be even more resistant to sulphite is *Zygosaccharomyces bailii* (Thomas & Davenport, 1985; Warth, 1985).

Little is known of the physiological basis for the different degrees of sulphite resistance among yeast species. Among strains of *S. cerevisiae*, differences in resistance have been attributed to production of compounds, particularly acetaldehyde, that bind sulphite to form α -hydroxysulphonates (Burroughs & Sparks, 1964), especially when the strains are grown in the presence of sulphite (Rankine, 1968; Rankine & Pocock, 1969; Weeks, 1969). Moreover, Stratford *et al.* (1987) attributed the greater sulphite resistance of a strain of *S. codes ludwigii* as compared with one of *S. cerevisiae* to its ability to produce greater amounts of acetaldehyde. The resistance of *S. codes ludwigii* was also caused in part, it was suggested (Stratford *et al.*, 1987), by its decreased ability to accumulate sulphite. The present paper compares the physiological basis of sulphite resistance in two strains each of *S. cerevisiae* and *Z. bailii*.

METHODS

Organisms. The yeasts used were *S. cerevisiae* NCYC 431, *S. cerevisiae* TC8 (Stratford & Rose, 1985), *Z. bailii* NCYC 563 and *Z. bailii* NCYC 1427. They were maintained at 4 °C on slopes of malt extract-yeast extract-glucose-mycological peptone (MYGP) agar (Wickerham, 1951).

Experimental cultures. Organisms were grown aerobically in a medium containing (l⁻¹): glucose, 20 g; (NH₄)₂SO₄, 3.0 g; KH₂PO₄, 3.0 g; yeast extract (Lab M), 1.0 g; MgSO₄ · 7H₂O, 30 mg; and CaCl₂ · 2H₂O, 30 mg (adjusted to pH 4.0 with HCl). This is the medium used by Stratford & Rose (1986) and is referred to as Medium A. It is, however, poorly buffered, and in experiments in which the yeasts were grown in the presence of sulphite it was replaced by Medium B which differed from Medium A in that KH₂PO₄ was omitted and replaced by 13.4 g K₂HPO₄ and 12.9 g citric acid. Under the conditions used, the pH value of cultures grown using Medium B did not fall below 4.0. Portions of medium (1 l) were dispensed into 2 l round flat-bottomed flasks which were plugged with cotton wool and sterilized by autoclaving at 6.89 × 10⁴ Pa for 10 min. Starter cultures (100 ml Medium A or B in 250 ml conical flasks) were inoculated with a pinhead of yeast from a slant culture and incubated at 30 °C for 24 h on an orbital shaker (200 r.p.m.). Portions of medium (1 l) were inoculated with portions of starter culture containing 0.05 mg dry wt *S. cerevisiae* NCYC 431, 0.5 mg dry wt *S. cerevisiae* TC8 or 1.0 mg dry wt of either of the *Z. bailii* strains. Growth was followed by measuring the optical density of portions of culture, measurements being related to dry wt of organism by a standard curve constructed for each strain of yeast. Organisms were harvested from mid exponential-phase cultures, containing 0.5 mg dry wt *S. cerevisiae* ml⁻¹ or 0.25 mg dry wt *Z. bailii* ml⁻¹, by filtration through a membrane filter (0.45 µm pore size; 50 mm diam.; Oxoid) and washed twice with 10 ml 30 mM-citrate buffer (pH 3.0).

Assessment of sulphur dioxide tolerance. The ability of the yeasts to grow in Medium B containing different concentrations of sulphite was measured using Dynatech Microplates. Organisms were harvested from mid exponential-phase cultures by centrifugation (12000 g for 2 min) and resuspended in fresh medium (pH 4.0) to give a suspension containing 0.1 mg dry wt ml⁻¹. Cell suspension (170 µl) was pipetted into each well of a microtitre plate leaving one well empty to use as a blank. Sodium metabisulphite (30 µl) diluted in fresh medium was added to each well giving final concentrations of sulphite ranging between zero and 3.3 mM across the plate. The blank well was filled with 200 µl water and the plate gently shaken for a few seconds to mix the suspensions. Replicate plates were prepared, covered, sealed in an airtight container with some moist tissue paper to minimize evaporation and incubated at 30 °C on an orbital shaker (200 r.p.m.). Using a Dynatech Microplate Reader (MR600), set at 600 nm, optical densities were measured at intervals up to 6 h after adjusting to zero against the blank well. Cells tended to settle to the bottom of the wells so the plates were gently agitated before optical densities were measured.

Measurement of sulphite accumulation. To measure initial velocities of sulphite accumulation, organisms grown in Medium A were washed twice with 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose, suspended in the same buffer at 10 mg dry wt ml⁻¹ and the suspension allowed to equilibrate for 3 min at 30 °C. A reaction mixture consisting of 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose and 10–200 µM-[³⁵S]sulphite (0.20 µCi ml⁻¹; 1 µCi = 37 kBq) was prepared in a universal bottle and warmed to 30 °C in a water-bath. Labelled sulphite was stored at –20 °C in 5 mM-EDTA under nitrogen gas in 0.5 ml portions (0.1 mCi ml⁻¹) to prevent oxidation. Portions (300 µl) of the suspension of organisms were dispensed into microcentrifuge tubes (Eppendorf). Using a 1.5 ml multi-dispense syringe pipette, 1.25 ml of labelled sulphite reaction mixture was added to the organisms and the suspension quickly mixed by refilling and emptying the syringe. After exactly 4 s, 1.5 ml of the suspension was rapidly filtered through a membrane filter (0.45 µm pore size; 25 mm diam.; Millipore) which had been washed with 5 ml 10 mM-sulphite in 30 mM-citrate buffer (pH 3.0). After filtration, three 1 ml portions of buffered sulphite solution of the same concentration as used in the experiment were used quickly to wash the organisms and filter. Filters with organisms were then placed in scintillation vials containing 7 ml Optiphase Safe (Fisons). Radioactivity in the vials was measured in an LKB Rackbeta liquid scintillation spectrometer (model 1217).

To measure the extent of sulphite accumulation, washed organisms grown in Medium A were suspended in glucose-containing citrate buffer as already described. Labelled sulphite was added to a suspension containing 2 mg dry wt ml⁻¹ giving a final concentration of 0.1–5.0 mM-sulphite (0.2 µCi ml⁻¹) and the suspension incubated at 30 °C. At appropriate time intervals, three 1 ml portions of suspension were filtered through prewashed filters as already described. The organisms were washed with three 1 ml portions of 30 mM-citrate buffer containing sulphite at the concentration used in the experiment. Radioactivity was measured as already described. Background activity was estimated by repeating the procedure without organisms to check washing efficiency and to make sure that sulphite was not binding to filters.

Measurement of plasma-membrane area of organisms. Dimensions of organisms were measured by observation in a light microscope fitted with an eyepiece graticule. In calculating membrane areas, it was assumed that organisms of *S. cerevisiae* were spheres and those of *Z. bailii* cylinders with rounded ends.

Measurement of intracellular water volume. Volumes of intracellular water in organisms in suspension were

calculated by measuring the differential distribution of $^3\text{H}_2\text{O}$, which equilibrates with both extracellular and intracellular water, and D-[1- ^{14}C]mannitol which is excluded by the plasma membrane. Preliminary experiments established that mannitol was not accumulated by any of the yeasts examined. To do this, washed organisms were suspended at 10 mg dry wt ml^{-1} in 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose and [^{14}C]mannitol at 0.01, 1.0 or 100 mM. The suspensions were incubated for 60 min at 30 °C and filtered through washed membrane filters (0.45 μm pore size; 50 mm diam.; Oxoid). The membranes were then washed with non-radioactive mannitol at the concentration used in the experiment, placed in scintillation vials containing 7 ml Optiphase Safe and radioactivity was measured as already described. To measure the volume of intracellular water, a suspension of washed organisms (10 mg dry wt ml^{-1}) grown in Medium A was prepared as already described. To 15 ml of suspension was added 10 mM-[^{14}C]mannitol (0.02 $\mu\text{Ci ml}^{-1}$) and 0.2 $\mu\text{Ci } ^3\text{H}_2\text{O ml}^{-1}$. Suspensions were incubated with continuous stirring at 4 °C for 10 min. Six 1 ml portions of suspension were then centrifuged in microcentrifuge tubes (Eppendorf) for 3 min at 12000 g . Duplicate 200 μl portions of supernatant from each tube were added to scintillation vials containing 7 ml Optiphase Safe and radioactivity was measured as previously described. Radioactivity in the suspension of organisms was measured by placing 12 200 μl portions of suspension in scintillation vials containing 7 ml Optiphase Safe.

Measurements of intracellular pH values. Intracellular pH values of organisms grown in Medium A were calculated by determining the equilibrium distribution of propionic acid across the plasma membrane (Conway & Downey, 1950). Washed organisms, suspended (5 mg dry wt ml^{-1}) in 30 mM-citrate buffer (9 ml) containing 100 mM-glucose, were allowed to equilibrate after adding 1 ml 0.1 mM-[2- ^{14}C]propionic acid (0.25 $\mu\text{Ci ml}^{-1}$) at 30 °C. After 1, 2, 4, 6, 8 and 10 min, duplicate 300 μl portions were taken from the suspension, rapidly filtered through washed membrane filters (0.45 μm pore size; 25 mm diam.; Millipore) and washed with 4 $\times$ 1 ml 0.01 mM-propionic acid at 4 °C. The filters with organisms were transferred to scintillation vials as already described. Once the time for equilibration had been ascertained, replicate measurements were obtained by sampling after 5 min incubation. Intracellular pH values were calculated from the expression derived by Waddell & Butler (1959):

$$\text{pH}_i = \text{pK}_i + \log_{10} [R(10^{(\text{pH}_e - \text{pK}_e)} + 1) - 1]$$

where $R = TA_i \cdot V_e / TA_e \cdot V_i$, pH_i and pH_e are the internal and external pH values, TA_i and TA_e the intracellular and extracellular total amounts of propionic acid, V_i and V_e the intracellular and extracellular volumes and pK_i and pK_e the dissociation constants for propionic acid in the internal and external environments. The internal and external dissociation constants for propionic acid were calculated from the Davies (1962) simplified version of the Debye-Hückel equations. Values for pK_i and pK_e were calculated to be 4.75 and 4.86, respectively.

Analytical methods. Free SO_2 was assayed by the method of Burroughs & Sparks (1964), which assumes that dissociation of bound SO_2 is minimized by lowering the pH value to 1.5. Acetaldehyde, glycerol and pyruvate were determined by using assay kits (Boehringer). Ethanol was determined by GLC as described by Beavan *et al.* (1982).

Chemicals. All reagents used were AnalaR or of the highest grade available commercially. Amersham supplied radioactively labelled chemicals

RESULTS

Effects of sulphite on growth

Sulphite inhibited growth of all four yeasts at concentrations up to and including 3.3 mM as assessed by the microplate method (Fig. 1). *Z. bailii* NCYC 563 was the most sensitive and *S. cerevisiae* NCYC 431 the least.

Accumulation of sulphite

Vertical Woolf-Eadie plots (Hofstee, 1959) were obtained with initial velocities of accumulation by all yeasts suspended in high concentrations of SO_2 (Fig. 2). However, at low concentrations of SO_2 and especially with *S. cerevisiae* NCYC 431, there was considerable deviation from the vertical. Equilibrium levels for accumulation of sulphite equivalents were reached somewhat faster with the strains of *S. cerevisiae* than with those of *Z. bailii* although all four strains had reached these levels after 10 min irrespective of the concentration of sulphite. As suspensions of organisms accumulated equilibrium levels of sulphite equivalents measured after 10 min incubation, intracellular pH values declined (Fig. 3). The greater the extent of accumulation of sulphite equivalents, the larger was the decline in internal pH value. Equilibrium accumulation values, and therefore decline in internal pH values, were smallest for *Z. bailii* NCYC 1427 (Fig. 3).

Fig. 1

Fig. 2

Fig. 1. Effect of sulphite concentration on growth of *S. cerevisiae* TC8 (○), *S. cerevisiae* NCYC 431 (●), *Z. bailii* NCYC 1427 (□) and *Z. bailii* NCYC 563 (■) in Medium B in microtitre wells. Values quoted are the means of measurements on eight separate plates. The maximum variation was $\pm 10\%$.

Fig. 2. Woolf-Eadie plots for accumulation of molecular SO_2 by *S. cerevisiae* TC8 (○), *S. cerevisiae* NCYC 431 (●), *Z. bailii* NCYC 1427 (□) and *Z. bailii* NCYC 563 (■) suspended in 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose at 30 °C. Concentrations of molecular SO_2 were calculated from the data of King *et al.* (1981). Bars indicate SD.

Fig. 3. Relationship between extent of accumulation of sulphite equivalents (open symbols) and intracellular pH values (closed symbols) in *S. cerevisiae* TC8 (a), *S. cerevisiae* NCYC 431 (b), *Z. bailii* NCYC 563 (c) and *Z. bailii* NCYC 1427 (d). Measurements were made after organisms had been suspended in buffer for 10 min. Values quoted are means of at least three determinations. Bars indicate SD.

Fig. 4. Effect of supplementing cultures of *S. cerevisiae* NCYC 431 (a) and *Z. bailii* NCYC 563 (b) with sulphite (■, control, △, 1.0 mM, ▲, 2 mM) on growth and ethanol formation. Also shown are the effects of these supplementations on concentrations of acetaldehyde (○), glycerol (●) and free sulphite (□) in culture supernatants. After supplementing cultures with sulphite, they were observed for a further 6 h. Values quoted are the means of three separate determinations. The maximum variation in values for concentrations of acetaldehyde and free sulphite was <10%; for concentrations of ethanol and glycerol the variation was $\pm 15\%$.

Production of binding compounds by organisms grown in the presence of sulphite

The effect of sulphite on growth of each of the yeasts in 1 litre cultures (Medium B) was assessed by adding the compound to mid exponential-phase cultures, and measuring the effect on density of organisms and on concentrations in culture filtrates of acetaldehyde, ethanol, glycerol, pyruvate and free sulphite over the following 6 h. Growth of *Z. bailii* NCYC 563 was

virtually completely inhibited following supplementation of cultures with 1.0 or 2.0 mM-sulphite (Fig. 4*b*). Ethanol production was also completely inhibited. Even in the supplemented cultures in which growth was almost completely inhibited, there was a decrease in the concentration of free sulphite despite the lack of production of acetaldehyde. Production of glycerol and of pyruvate (not shown), which was detectable in unsupplemented cultures, was also completely inhibited. A very similar pattern of responses was observed in cultures of *S. cerevisiae* TC8 (data not shown). The much greater production of glycerol by this strain in unsupplemented cultures, which reached a concentration of approximately 7 mM in 6 h cultures, was also completely inhibited by supplementation with 1.0 or 2.0 mM-sulphite. Supplementing cultures of *S. cerevisiae* NCYC 431 with 1.0 mM-sulphite had no effect on growth or ethanol production (Fig. 4*a*). In these cultures, the concentration of free sulphite declined rapidly, while there was an increased production of glycerol and rapid appearance of acetaldehyde in culture filtrates. When cultures of this yeast were supplemented with 2.0 mM-sulphite, growth was decreased considerably and this was accompanied by decreased production of ethanol and glycerol (Fig. 4*a*). However, there was again a rapid decline in the concentration of free sulphite, which was accompanied by a greater increase in acetaldehyde concentration than was observed in cultures supplemented with 1.0 mM-sulphite. Again, production of pyruvate was unaffected (not shown). Cultures of *Z. bailii* NCYC 1427 showed a very similar pattern of responses to those of *S. cerevisiae* NCYC 431 (data not shown), except that less glycerol was produced in unsupplemented cultures while supplementation with 1.0 mM-sulphite lowered glycerol production.

DISCUSSION

The two strains of *S. cerevisiae* used to compare sulphite resistance with strains of *Z. bailii*, which have been reported to be extremely resistant to the compound (Thomas & Davenport, 1985; Warth, 1985), were selected without any knowledge of their reaction to sulphite. *S. cerevisiae* NCYC 431 is a strain originating from a distillery, and has a high tolerance of ethanol (Cartwright *et al.*, 1986, 1987), while *S. cerevisiae* TC8 is a strain used in cider-making and which has been reported to excrete H₂S (Stratford & Rose, 1985). It was surprising, therefore, to find that, of the four strains examined, one of *S. cerevisiae* was the most tolerant to sulphite while a strain of *Z. bailii* was the most sensitive. The availability of authenticated strains of *Z. bailii* is limited. *Z. bailii* NCYC 563 was included in the survey because it has been used in research into sulphite resistance of spoilage yeasts (Cole *et al.*, 1987). Significantly, it was the least resistant of the strains examined in the present study.

Two yeasts, namely *S. cerevisiae* (Stratford & Rose, 1986) and *S. codes ludwigii* (Stratford *et al.*, 1987), have been shown to transport SO₂ by free diffusion, based on evidence from vertical Woolf-Eadie plots. The present report shows that passage of SO₂ into strains of *Z. bailii* is also by free diffusion. It was also interesting to note that the deviation from verticality, observed in the present study with strains of *Z. bailii* and previously with *S. cerevisiae* TC8 (Stratford & Rose, 1986) and *S. codes ludwigii* (Stratford *et al.*, 1987), was very much more pronounced with *S. cerevisiae* NCYC 431. This suggests that, at low concentrations of SO₂, a facilitated transport system operates, possibly to transport the HSO₃⁻ ion. With vertical Woolf-Eadie plots, the value at the intercept on the abscissa is equivalent to the permeability coefficient for passage of SO₂ into the organism (Laidler, 1977). It is clear, therefore, that the two strains of *Z. bailii* have lower permeability coefficients than either of the *S. cerevisiae* strains.

Our discovery of a correlation between ability of yeasts to grow in the presence of sulphite and sulphite-induced production of acetaldehyde suggests that production of this sulphite-binding compound contributes significantly to the resistance. It is also noteworthy that the two most sulphite-resistant yeasts examined, namely *S. cerevisiae* NCYC 431 and *Z. bailii* NCYC 1427, are able to produce large amounts of acetaldehyde when growth was almost completely inhibited by 2.0 mM-sulphite. Excretion of acetaldehyde together with glycerol in cultures of *S. cerevisiae* supplemented with sulphite has been known for many years (Neuberg & Reinfurth, 1918, 1919), and constitutes Neuberg's second form of fermentation (Nord & Weiss, 1958). Our data are in general agreement with the finding of Neuberg & Reinfurth (1919) that, in the presence of

sulphite, acetaldehyde and glycerol are produced in equimolar amounts by strains of *S. cerevisiae*. Moreover, the data show for the first time that this is true also for strains of *Z. bailii*. Production of glycerol by *Z. acidifaciens* (now recognized as *Z. bailii*) was reported by Nickerson & Carroll (1945).

When SO_2 enters the yeast cell, it encounters an environment which is around pH 6.5 with the result that a large proportion of the SO_2 is converted into HSO_3^- . This explains the ability of yeasts to concentrate sulphite intracellularly. At the same time, the intracellular pH value declines, which in turn lowers the transmembrane pH gradient and hence dissipates the proton-motive force across the plasma membrane. A result of this would be to retard or inactivate processes, such as active transport of solutes, that require energy from the proton-motive force. The discovery that the decrease in internal pH value following accumulation of sulphite is not of the same magnitude in all strains of yeast suggests that the internal buffering capacity of organisms might be important in sulphite resistance. While invoking a role for energy metabolism in sulphite resistance of yeasts, it is worth noting that exposure of *S. cerevisiae* to sulphite leads to a rapid decrease in the content of ATP (Schimz & Holzer, 1979) which has been attributed primarily to the action of sulphite on the enzyme glyceraldehyde-3-phosphate dehydrogenase (Hinze & Holzer, 1986).

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