SHORT COMMUNICATION

Water Stress Plating Hypersensitivity of Yeasts

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Saccharomyces cerevisiae, when growing exponentially in batch culture, passed through a phase in which, on average, one cell in 10^4 survived plating onto a low water activity (a_w) agar medium. Stationary phase cultures were resistant as were all other species tested, with the exception of *Candida krusei*. In continuous culture, *S. cerevisiae* was more resistant at low than at high dilution rates. Plating at low a_w was lethal to those cells that were not protected by an adequate content of compatible solute. In naturally resistant yeasts and in *S. cerevisiae* that had been exposed to an adaptation process, the compatible solute was one or more types of polyhydric alcohol. Resistance in stationary phase was attributable to a different cause.

INTRODUCTION

Edgley & Brown (1983) reported major differences in the process of adaptation from a conventional broth medium to one containing NaCl (10%, w/v) between *Saccharomyces cerevisiae* and the xerotolerant species *Saccharomyces rouxii*. The differences included a dramatic drop in apparent viability by the former yeast but not by the latter. The apparent loss in viability by *S. cerevisiae* complicated interpretation of the adaptation sequence, in particular whether adaptation was achieved essentially by the whole population or a small residue that survived during the transition. The present paper describes a peculiarity of population dynamics that is relevant to that question. We have called this phenomenon 'water stress plating hypersensitivity'.

METHODS

Organisms. *S. rouxii* and *S. cerevisiae* were maintained as previously described (Edgley & Brown, 1983). *Candida albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis* were obtained from W. Crozier (Wollongong Hospital, Australia); *S. bailii* and *Pichia membranaefaciens* from J. Pitt (CSIRO Food Research Labs, North Ryde, Australia); *P. miso* from H. Onishi (Dept. of Agricultural Chemistry, Kagoshima University, Japan) and *Debaryomyces hansenii* from L. Adler (Dept. of Marine Microbiology, University of Gothenburg, Sweden). *Saccharomyces carlsbergensis* and *S. cerevisiae* var. *ellipsoideus* were from our own culture collection. For experimental purposes, liquid batch cultures were grown as previously described (Edgley & Brown, 1983) in basal yeast medium (BYM), a conventional high a_w broth, or with modifications as indicated in Results.

Counting. Samples for total counts and plating were first treated in a glass/Teflon homogenizer (diameter 16 mm) to disaggregate clumps. A sample of 3 ml was subjected to 75 double strokes of the Teflon plunger (about 2 min). Total counts were made conventionally in a counting chamber. A colony-forming unit (c.f.u.) is defined as any cell or aggregate of cells which, if viable, would produce a single colony.

Plating media were used according to Edgley & Brown (1983) except that pour plates were used instead of drop plates. This change was introduced because yeast suspensions adapted to a lower a_w during the drying of a drop on

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Abbreviations: BYM, basal yeast medium; MA, malt agar; SHA, synthetic honey agar.

a 'synthetic honey agar' plate (SHA, containing 48%, w/v, glucose, a_w 0.924). Plates were seeded with 0.1 ml suspension and poured with 10 ml agar. They were incubated at 30 °C inside plastic bags to restrict evaporation. There were no qualitative differences of consequence between results obtained with a plating medium containing NaCl (8 or 10%, w/v) and one with 48% glucose (SHA; see Fig. 1). SHA was used routinely since colonies could be counted after 6 d compared with about three weeks for NaCl agar.

Polyol chromatography. Freeze-dried yeast was extracted with ethanol (Edgley & Brown, 1983). Polyols were analysed by thin-layer chromatography on silica gel with propan-1-ol/concentrated NH_4OH /water (6:2:1, by vol.) and with butan-1-ol/acetic acid/water (6:1:2, by vol.) as developing solvents. They were detected with a periodate-Schiff base reagent (Baddiley *et al.*, 1956) and with alkaline silver nitrate. Glycerol was estimated as described by Edgley & Brown (1983).

RESULTS AND DISCUSSION

General characteristics

During the course of a growth cycle in BYM, *S. cerevisiae* gave plate counts on malt agar (MA) and various low a_w stressing agars as shown in Fig. 1. The essential characteristic of the phenomenon was a huge drop in colony-forming ability on a stressing agar during the period of exponential growth. The population developed resistance as it entered stationary phase. Since stationary phase inocula were always used, the culture was also resistant at the beginning of the growth cycle.

At its maximum, the plating discrepancy (log MA count – log stressing agar count) was, for Fig. 1, about 2.5–4 but, under some conditions, discrepancies up to 6 were obtained. In other words, 1 c.f.u. in 10^6 is viable on the stressing agar under such extreme conditions. Plate counts on NaCl agar were not increased by inclusion in the agar of glycine betaine (0.25–0.5 mM) or glycerol (0.5–1.0 M), agents capable of relieving solute stress under some conditions (cf. Le Rudulier *et al.*, 1984). Since early results indicated a close correlation between plating discrepancy and budding, we considered a mechanism in which a specific step in the cell division cycle was critically sensitive to the solute stress. The correlation was subsequently found to be superficial and strongly dependent on experimental conditions. When *S. cerevisiae* was grown in BYM in continuous culture, the plating discrepancy was about 1 for dilution rates in the range 0.02–0.05 h^{-1} and about 3 in the range 0.1–0.13 h^{-1} with a transition between the ranges.

Distribution

Fourteen species and strains (see Methods) were screened. Plating hypersensitivity was found only in strains of *S. cerevisiae*, including var. *ellipsoideus*, and in *Candida krusei*. None of the sensitive yeasts, when harvested from mid-exponential phase in BYM, had accumulated polyols that were detected on chromatograms [≥ 0.02 mmol (g dry yeast) $^{-1}$, for glycerol]. Every resistant yeast accumulated one or more of the polyols glycerol, erythritol, arabitol or mannitol in readily detectable quantities. Accumulation of arabitol by *D. hansenii* and *S. rouxii* had already been established (Adler & Gustafsson, 1980; Brown, 1978). Both haploid and diploid strains were included in the comparison; ploidy did not correlate with sensitivity.

Physiological characteristics

The heterogeneity of the population responsible for the results in Fig. 1 was physiological, not genetic, inasmuch as resistant colonies isolated from mid-exponential phase SHA plates gave rise to cultures with identical characteristics. Moreover, failure to grow on SHA was attributable to death, not dormancy. This was demonstrated by two versions of an experiment in which either excised areas or whole plates of SHA were incubated in quarter-strength Ringer's solution at various time intervals after the plate was poured. Comparisons with appropriate controls showed clearly that, by this criterion, those cells that did not form colonies had died and were not merely suppressed.

The relation between plating discrepancy and a_w (or, in Fig. 2, glucose concentration) was complex. There was no discrepancy below 30% (w/v) glucose but there was approximate proportionality between the two variables at higher glucose concentrations (Fig. 2).

Fig. 1

Fig. 1. Partial growth cycle of *Saccharomyces cerevisiae*. (a) Proportion of c.f.u. with buds. (b) Population counts: ■, total c.f.u.; ○, MA colony count; □, SHA colony count; ◆, colony count on NaCl agar (8% w/v, NaCl); ▲, colony count on NaCl agar + glycine betaine (0.5 mM); △, colony count on NaCl agar + glycerol (0.5 M).

Fig. 2

Fig. 2. Plating discrepancy as a function of glucose concentration in the stressing agar. The various symbols denote different experimental cultures. Inocula were taken from mid-exponential phase cultures in BYM.

Growth of *S. cerevisiae* in BYM containing NaCl eliminated plating hypersensitivity but there was a pronounced effect of NaCl concentration. There was no plating discrepancy with cultures grown in media containing 2% (w/v) or more NaCl; below 2%, plating discrepancy increased sharply with decreasing NaCl concentration. Furthermore, washing (three times by centrifugation in 0.01 M-potassium phosphate, pH 6.5) substantially reversed the adaptation. Adaptation was accompanied by accumulation of glycerol (from $<0.05 \text{ mmol g}^{-1}$ for yeast grown in BYM to 4 mmol g^{-1} when grown in 4% NaCl). Washing removed 80% of the glycerol from yeast grown in 2% NaCl. The yeast also adapted within about 1 h when incubated in a simple NaCl solution (i.e. no nutrient) at concentrations of 1–4% (w/v). Here, too, adaptation was complete in 2% NaCl. Adaptation was thus a bulk phenomenon, not selection of an inherently resistant variant.

Moreover, the amount of intracellular glycerol that accumulated in response to growth in 2% NaCl (about $0.99 a_w$) was substantially less than that needed for complete osmotic adjustment to SHA ($0.924 a_w$). This suggests that the protection conferred by the accumulated glycerol was achieved primarily through its role as a compatible solute rather than by the osmoregulatory process of promoting complete turgor adjustment.

The inherent resistance of the insensitive strains and the acquired resistance of *S. cerevisiae* on adaptation are each attributable to polyol accumulation and the function of such compounds as compatible solutes. The resistance developed by *S. cerevisiae* in stationary phase is largely, but probably not wholly, attributable to the accumulation of the storage polysaccharide trehalose (Brown *et al.*, 1986); this is not an osmoregulatory process.

Water stress plating hypersensitivity is strikingly similar in several respects to the adaptation of *Escherichia coli* to NaCl as described by Doudoroff (1940).

REFERENCES

- ADLER, L. & GUSTAFSSON, L. (1980). Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. *Archives of Microbiology* **124**, 123–130.
- BADDILEY, J., BUCHANAN, J. G., HANDSCHUMACHER, R. E. & PRESCOTT, J. F. (1956). Chemical studies in the biosynthesis of purine nucleotides. I. Preparation of N-glycylglycosylamine. *Journal of the Chemical Society*, 2818–2823.
- BROWN, A. D. (1978). Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Advances in Microbial Physiology* **17**, 181–242.
- BROWN, A. D., MACKENZIE, K. F. & SINGH, K. K. (1986). Selected aspects of microbial osmoregulation. *FEMS Microbiology Reviews* **39** (in the Press).
- DOUDOROFF, M. (1940). Experiments on the adaptation of *Escherichia coli* to sodium chloride. *Journal of General Physiology* **23**, 585–611.
- EDGLEY, M. & BROWN, A. D. (1983). Yeast water relations: physiological changes induced by solute stress in *Saccharomyces cerevisiae* and *Saccharomyces rouxii*. *Journal of General Microbiology* **129**, 3453–3463.
- LE RUDULIER, D., STRØM, A. R., DANDEKAR, A. M., SMITH, L. T. & VALENTINE, R. C. (1984). Molecular biology of osmoregulation. *Science* **224**, 1064–1068.