Applied and Environmental Microbiology, Dec. 2003, p. 7032–7034 Vol. 69, No. 12
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.12.7032–7034.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Compatible Solutes Protect against Chaotrope (Ethanol)-Induced, Nonosmotic Water Stress

John E. Hallsworth,[1,2]* Bernard A. Prior,[3] Yoshiyuki Nomura,[1] Masayoshi Iwahara,[1] and Kenneth N. Timmis[2]

Department of Applied Microbial Technology, Sojo University, Kumamoto City, Japan[1]; Department of Biological Sciences, University of Essex, Colchester, United Kingdom[2]; and Department of Microbiology, University of Stellenbosch, Matieland, South Africa[3]

Received 19 February 2003/Accepted 11 September 2003

Water stress is one of the major stresses experienced by cellular systems and can take a number of distinct forms. In response to turgor-related osmotic stress, cells produce compatible solutes that are macromolecule protectants and also counteract the outflow of water from stressed cells. In this report we show that the germination of conidia of *Aspergillus nidulans*, a sensitive indicator of water stress, in the presence of ethanol is correlated with the intracellular concentration of the compatible solutes glycerol and erythritol, which protect against both osmotic and nonturgor forms of water stress.

Cellular systems consist of up to 97% water, so thermodynamic changes in water availability cause fundamental biological stress that can severely impact vital metabolic processes (3). Water stress can be induced by a net loss of water from cells, e.g., due to high levels of extracellular solutes such as NaCl that reduce cell turgor and induce osmotic stress. Cells exposed to such agents are subjected to both reduced turgor and stabilizing effects on macromolecules, which can lead to membrane rigidity and impairment of protein structure (3, 16, 19, 25). Intracellular water activity also may be reduced by chaotropic compounds, such as ethanol, that decrease the strength of hydrogen bonding and other electrostatic interactions and thereby perturb the structure and function of hydrated macromolecules, including nucleic acids, proteins, and lipids.

Cells generally respond to osmotic stress by acquiring and/or producing compatible solutes that protect macromolecule structure and at the same time increase the osmotic pressure of the cytoplasm and thereby counteract water loss from cells (1, 3). Our working hypothesis is that these compatible solutes also protect against intracellular water stress due to alterations in the hydration of cellular macromolecules caused by ethanol, a chaotropic solute that does not cause osmotic stress.

The ascomycete *Aspergillus* (*Emericella*) *nidulans* is a model microorganism whose genetics and metabolism, including those related to stress and toxicology, have been widely studied. *A. nidulans*, like most yeast and fungal cells, synthesizes low-molecular-weight polyols, such as glycerol, in response to osmotic stress (3, 21). Our objective in this study was to determine whether increased intracellular levels of glycerol and erythritol affect conidial germination in *A. nidulans* when the organism is cultured under conditions that reduce cell turgor and induce osmotic stress (e.g., in a high-NaCl environment), as well as those that increase intracellular water stress by reducing the strength of water-macromolecule interactions (e.g., at high ethanol concentrations).

* Corresponding author. Mailing address: Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, United Kingdom. Phone: 44-1206 872535. Fax: 44-1206 872592. E-mail: jehalls@essex.ac.uk.

MATERIALS AND METHODS

Organism, media, and production of conidia. A culture of *A. nidulans* (IFO 4342) was obtained from the Institute for Fermentation Osaka (Osaka, Japan) and maintained on potato dextrose agar (PDA; Nissui, Tokyo, Japan) at 25°C. All media were buffered with 21.3 g of 2-(*N*-morpholino)ethanesulfonic acid (MES) liter^{-1}, and the pH was adjusted to 5.8 with 2.5 M NaOH. The water activity (a_w) values of the media were determined by using a Humidat-IC II apparatus (Novasina, Talstrasse, Switzerland) fitted with a conventional humidity sensor and a protective eVALC alcohol filter (Novasina) (13). Three measurements of medium a_w were taken at 25°C for one sample from each independent experiment, and the a_w variation was within $\pm$0.003.

The media used were PDA (control), PDA plus 180 g of glycerol liter^{-1}, and PDA plus 115 g of KCl liter^{-1} (Table 1). The glycerol and KCl concentrations employed were based on those used in previous studies (7, 8). Petri plates were inoculated with 2-mm-diameter plugs of agar taken from the periphery of an exponential-phase culture growing on medium of the same composition. Aerial conidia were obtained from these cultures by incubation for 4 days (for cultures on PDA) or 8 days (for cultures on PDA plus glycerol and on PDA plus KCl) at 25°C. Plates of each medium were kept together in a sealed bag of low-density polyethylene to maintain a constant relative humidity and the medium a_w while allowing gaseous exchange (17, 20). Conidia from each medium were harvested into sterile AnalaR water (Merck, Poole, United Kingdom), the suspension was filtered through glass wool to remove any hyphal fragments, and the conidia were washed and freeze-dried as described previously (7).

Extraction and analysis of compatible solutes. Freeze-dried conidia (10 mg) were resuspended in 1 ml of AnalaR water in a 2-ml microcentrifuge tube, sonicated (for 120 s at an amplitude of 28 μm), placed in a boiling water bath (5.5 min), and filtered (10). Polyol-containing filtrates were injected onto a series 4500 high-pressure liquid chromatograph (Dionex Corp., Sunnyvale, Calif.) fitted with a CarboPac PA1 column (Dionex) and quantified by pulsed electrochemical detection (10). Analyses were performed in triplicate with conidia harvested from three separate experiments that had been carried out at different times.

Assessment of germination. Germination on PDA (control; 0.998 a_w), PDA plus NaCl, and PDA plus ethanol was assessed over a range of concentrations in six-vented disposable petri plates (90 by 15 mm). At 25°C, the a_w values of PDA plus ethanol were (percentages signify weight per volume) 0.977 (4%), 0.975 (4.5%), 0.973 (5%), 0.971 (5.5%), 0.969 (6%), 0.967 (6.5%), 0.961 (7%), 0.957 (7.5%), and 0.955 (8%), and the a_w values of PDA plus NaCl were 0.975 (8%), 0.968 (10%), 0.960 (12%), 0.953 (14%), 0.947 (16%), 0.940 (18%), 0.934 (20%), and 0.930 (22%). Ethanol was incorporated into media that had cooled to <50°C after sterilization to avoid losses through evaporation.

Conidia were harvested into sterile distilled water (if germination was assessed

TABLE 1. Intracellular polyols from conidia produced on different media

Medium composition[b]	Medium a_w	Polyol concn (mg g of dry conidia^{-1})[a]		
		Glycerol	Erythritol	Mannitol[c]
PDA (control)	0.998	0.84	2.1	48
PDA + 180 g of glycerol liter^{-1}	0.939	63	27	44
PDA + 115 g of KCl liter^{-1}	0.930	3.5	6.4	45
LSD[d]		0.9	2.2	4.3

[a] Trace amounts of arabitol were also detected (data not shown).
[b] All based on PDA plus 21.3 g of MES buffer liter^{-1}.
[c] Mannitol is included to show that its intracellular concentration did not vary significantly between conidia from different media and for consistency with other studies of intracellular polyols in conidia (7–10).
[d] Least significant difference between values obtained from conidia produced on different media ($P < 0.05$).

on PDA) or into sterile distilled water containing ethanol or NaCl at the concentration corresponding to that in the germination medium used, centrifuged, washed, and resuspended in the wash solution, as described previously (7). Petri plates of each medium were inoculated with 200 μl of the conidial suspension (which was spread with a glass rod, giving approximately 5,000 conidia cm^{-2}), sealed in polyethylene bags, and incubated at 25°C. Two 9-mm-diameter disks were removed from each plate at intervals of approximately 6 h and stained with lactophenol cotton blue, and the germination status of 100 conidia from each treatment (three replicates) was assessed by light microscopy. Conidia with germ tubes longer than their diameter were considered to have germinated. Thirty well-separated germinated conidia from each treatment (three replicates) were evaluated for germ tube length, number of germ tubes per conidium, and diameter of imbibed spores.

RESULTS

Compatible-solute contents of conidia. Conidia contained three main polyols, glycerol, erythritol, and mannitol (Table 1), as well as trace amounts of arabitol (<1 mg g of dry conidia^{-1}). The culture medium used did not affect the concentration of mannitol in conidia (44 to 48 mg g^{-1}) but did affect the glycerol and erythritol contents of conidia (Table 1). Conidia from *A. nidulans* cultures grown on PDA (low-compatible-solute conidia) contained negligible amounts of glycerol and erythritol, whereas those produced on PDA plus glycerol (high-compatible-solute conidia) contained 90 mg of glycerol plus erythritol g^{-1} (dry weight). Conidia obtained from *A. nidulans* grown on PDA plus KCl contained glycerol and erythritol concentrations that were low but higher than control concentrations (Table 1).

Germination and germ tube growth of conidia in relation to polyol content. Germination on PDA without stressors was rapid; by 6.5 h, the majority of control (low-compatible-solute) conidia had imbibed water and swollen, and 5% had already germinated (data not shown). By 12 h, almost 90% had germinated, and the mean length of the primary germ tubes had reached 34 μm (Table 2). In contrast, by 48 h, only 4% of low-compatible-solute conidia had germinated on media containing 6.5% (wt/vol) ethanol, and even fewer had germinated on media containing ≥7% (wt/vol) ethanol, despite extensive conidial swelling. For the conidia that did germinate, the mean lengths of the germ tubes and the proportion of conidia with multiple germ tubes decreased with increasing ethanol concentration in the medium. Low-compatible-solute conidia also failed to germinate on medium containing ≥16% (wt/vol) NaCl (Table 2).

Conidia containing large amounts of glycerol and erythritol germinated only marginally more quickly on PDA than did control conidia (Table 2). However, these high-compatible-solute conidia germinated rapidly on media containing ethanol or NaCl; by 12 h, they had begun to germinate on media containing 4% (wt/vol) ethanol or 8% (wt/vol) NaCl, and by 34 h, the majority had germinated on media containing 5.5%

TABLE 2. Influence of water stressors on germination parameters of conidia containing different levels of compatible solutes[a]

Water stressor and % (wt/vol)	Germination (%)				Conidia with multiple germ tubes (%)				Mean germ tube length[d]				Swollen conidia[e] (%)			
	Conidial polyol content[b]			LSD[c]	Conidial polyol content			LSD	Conidial polyol content			LSD	Conidial polyol content			LSD
	Low	Int	High		Low	Int	High		Low	Int	High		Low	Int	High	
Ethanol																
0	88	87	91	10	36	54	48	17	34	31	37	8.8	95	96	94	14
6.5	4.1	45	67	5.9	21	69	93	10	23	30	33	4.3	89	94	95	11
7	0.2	47	85	3.8	0	91	96	6.3	8.7	15	24	3.0	85	86	89	9.8
7.5	0.7	36	45	2.4	0	21	42	7.1	7.7	14	16	3.9	83	92	91	6.1
8	0	0	1.6		0	0	0		0	0	7.2		1.2	26	82	6.3
NaCl																
0	87	92	90	9.3	34	37	35	7.2	27	24	27	5.2	97	90	94	11
14	93	90	95	7.2	61	66	82	6.6	78	84	99	6.5	93	96	95	14
16	0	57	81	13	0	39	82	9.3	0	35	37	5.9	90	82	85	6.9
18	0	0.1	3.2	0.5	0	0	34		0	0	4.3		2	64	90	8.1
20	0	0	0		0	0	0		0	0	0		0	16	56	7.3

[a] Ethanol and NaCl were added to germination medium that consisted of PDA plus 21.3 g of MES buffer liter^{-1}. Data for germination on PDA only (no ethanol or NaCl) were collected 12 h after inoculation, and all other data (on medium containing ethanol or NaCl) were collected after 48 h.
[b] Low for conidia from PDA, intermediate (Int) for those from PDA plus KCl, and high for conidia from PDA plus glycerol; see Table 1.
[c] Least significant difference (LSD) between values obtained from conidia germinated on different media ($P < 0.05$).
[d] Of the longest germ tube.
[e] Swollen conidia consist of the sum of those that had both swollen and germinated and those that had swollen but not yet produced a germ tube.

(wt/vol) ethanol or 14% (wt/vol) NaCl (data not shown) and germ tube growth was proportionally advanced. By 48 h, most conidia had germinated on media containing all but the highest concentrations of ethanol or NaCl, and even the conidia at these highest concentrations had imbibed water and were apparently preparing to germinate (Table 2). Conidia from cultures grown on PDA plus KCl, the medium with the lowest a_w, were slightly more resistant to ethanol and salt stress than those grown on unmodified PDA but substantially less resistant to both stressors than high-compatible-solute conidia produced on PDA plus glycerol (Table 2), eliminating a_w of the culture medium as a possible variable affecting the observed differences in germination.

DISCUSSION

Studies of water stress in microorganisms have focused mainly on turgor-related (osmotic) stress caused by kosmotropic solutes (3) such as polyethylene glycol 600 and NaCl. In such cases, microbial cells synthesize and/or accumulate low-molecular-weight compatible solutes (3). Glycerol and erythritol enhance conidial germination of several fungal species under NaCl-induced osmotic stress (8) and also have this effect on *A. nidulans* (Table 2). In contrast, mannitol is relatively ineffective as an osmoprotectant (2, 4, 7, 8).

In response to chaotropic solutes (e.g., LiCl, urea, and ethanol), microbial cells also up-regulate proteins involved in protein stabilization, lipid metabolism and membrane structure, protein synthesis, and energy metabolism (14). Ethanol and other chaotropes readily traverse lipid bilayers (5, 6, 15, 23) but nevertheless reduce water activity and decrease electrostatic interactions in biological macromolecules (see Materials and Methods) (14, 16, 19, 25). We found that compatible solutes protect against ethanol (Table 2), which is consistent with the hypothesis that ethanol's primary effects on microbial metabolism are through perturbation of water-macromolecule relations. Glycerol and erythritol also reduce the adverse effects of ethanol on enzymes and membranes in vitro (18, 22). There is some evidence that the amino acid proline may play the same role in some microbial cells, such as those of *Saccharomyces cerevisiae* (3, 11, 24).

In conclusion, the osmoprotectants glycerol and erythritol protect against ethanol-induced water stress in *A. nidulans.* These results are consistent with the hypothesis that compatible solutes are general protective agents against different forms of water stress and extend our previous observations that microbial growth and metabolism are inhibited by chaotrope-induced water stress (11, 12, 14). These results also raise the question of whether the principal stress mediated by both chaotropic and kosmotropic solutes is the same, namely, a perturbation of water-macromolecule relations.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Education, Science, Sports and Culture of Japan; the Sagawa Foundation for Promotion of Frontier Science, Kumamoto, Japan; and MIFRIEND (EU). J. E. Hallsworth gratefully acknowledges the receipt of a postdoctoral fellowship from the Japan Society for the Promotion of Science, Tokyo, Japan.

We thank K. Kayata and K. Kawashita (Sojo University, Kumamoto, Japan) for providing technical assistance and P. Nicholls and T. J. McGenity (University of Essex, Essex, United Kingdom) and S. Rainieri (Suntory Limited, Osaka, Japan) for helpful discussions.

REFERENCES

1. **Arakawa, T., and S. N. Timasheff.** 1985. The stabilization of proteins by osmolytes. Biophys. J. **47:**411–414.
2. **Brown, A. D.** 1978. Compatible solutes and extreme water stress in eukaryotic micro-organisms. Adv. Microb. Physiol. **17:**181–242.
3. **Brown, A. D.** 1990. Microbial water stress physiology. John Wiley and Sons, Chichester, United Kingdom.
4. **Chirife, J., G. Favetto, and C. Ferro Fontán.** 1984. Microbial growth at reduced water activities: some physicochemical properties of compatible solutes. J. Appl. Bacteriol. **56:**259–268.
5. **Giocondi, M. C., and C. Le Grimellec.** 1991. Water permeation in Madin-Darby canine kidney cells is modulated by membrane fluidity. Biochim. Biophys. Acta **1064:**315–320.
6. **Guijarro, J. M., and R. Lagunas.** 1984. *Saccharomyces cerevisiae* does not accumulate ethanol against a concentration gradient. J. Bacteriol. **160:**874–878.
7. **Hallsworth, J. E., and N. Magan.** 1994. Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. Microbiology **140:**2705–2713.
8. **Hallsworth, J. E., and N. Magan.** 1995. Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. Microbiology **141:**1109–1115.
9. **Hallsworth, J. E., and N. Magan.** 1996. Culture age, temperature and pH affect the polyol and trehalose contents of fungal propagules. Appl. Environ. Microbiol. **62:**2435–2442.
10. **Hallsworth, J. E., and N. Magan.** 1997. A rapid HPLC protocol for detection of polyols and trehalose. J. Microbiol. Methods **29:**7–13.
11. **Hallsworth, J. E.** 1998. Ethanol-induced water stress in yeast. J. Ferment. Bioeng. **85:**125–137.
12. **Hallsworth, J. E., Y. Nomura, and M. Iwahara.** 1998. Ethanol-induced water stress and fungal growth. J. Ferment. Bioeng. **86:**451–456.
13. **Hallsworth, J. E., and Y. Nomura.** 1999. A simple method to determine the water activity of ethanol-containing samples. Biotechnol. Bioeng. **62:**242–245.
14. **Hallsworth, J. E., S. Heim, and K. N. Timmis.** Chaotropic solutes cause water stress in *Pseudomonas putida.* Environ. Microbiol., in press.
15. **Hetter, D., and H. Wang.** 1989. Protein release from *Escherichia coli* cells permeabilized with guanidine-HCl and Triton X100. Biotechnol. Bioeng. **33:**886–895.
16. **Hribar, B., N. T. Southall, V. Vlachy, and K. A. Dill.** 2002. How ions affect the structure of water. J. Am. Chem. Soc. **124:**12302–12311.
17. **Huang, M. R., S. X. Li, Z. Q. Dong, W. Feng, X. Y. Wang, S. Y. Gu, Y. H. Wu, and X. A. Huang.** 2002. Oxygen enrichment from air through multilayer thin low-density polyethylene films. J. Appl. Polymer Sci. **83:**3013–3021.
18. **Ivanov, I. T.** 2001. Rapid method for comparing the cytotoxicity of organic solvents and their ability to destabilize proteins of the erythrocyte membrane. Pharmazie **56:**808–809.
19. **Koynova, R., J. Brankov, and B. Tenchkov.** 1997. Modulation of lipid phase behavior by kosmotropic and chaotropic solutes—experiment and thermodynamic theory. Eur. Biophys. J. Biophys. Lett. **25:**261–274.
20. **Laguna, M. F., J. Guzman, and E. Riande.** 2001. Transport of carbon dioxide in linear low-density polyethylene determined by permeation measurements and NMR spectroscopy. Polymer **42:**4321–4327.
21. **Redkar, R. J., R. D. Locy, and N. K. Singh.** 1995. Biosynthetic pathways of glycerol accumulation under salt stress in *Aspergillus nidulans.* Exp. Mycol. **19:**241–246.
22. **Simon, L. M., M. Kotorman, G. Garab, and I. Laczko.** 2002. Effects of polyhydroxy compounds on the structure and activity of alpha-chymotrypsin. Biochem. Biophys. Res. Commun. **293:**416–420.
23. **Takahashi, H., A. Matsuo, and I. Hatta.** 2000. Effects of chaotropic and kosmotropic solutes on the structure of lipid cubic phase: monoolein-water systems. Mol. Cryst. Liq. Cryst. **347:**475–482.
24. **Thomas, K. C., S. H. Hynes, and W. M. Ingledew.** 1994. Effects of particulate materials and osmoprotectants on very-high-gravity ethanolic fermentation by *Saccharomyces cerevisiae.* Appl. Environ. Microbiol. **60:**1519–1524.
25. **Wiggins, P. M.** 2001. High and low density intracellular water. Cell. Mol. Biol. **47:**735–744.