Environmental Microbiology (2003) doi:10.1046/j.1462-2920.2003.00478.x

Chaotropic solutes cause water stress in *Pseudomonas putida*

John E. Hallsworth,[1]* Sabina Heim[2] and Kenneth N. Timmis[1,2]

[1]*Department of Biological Sciences, University of Essex, Colchester, CO4 3SQ, UK.*

[2]*Division of Microbiology, GBF – National Research Centre for Biotechnology, Braunschweig, D-38124, Germany.*

Summary

Low water availability is the most ubiquitous cause of stress for terrestrial plants, animals and microorganisms, and has a major impact on ecosystem function and agricultural productivity. Studies of water stress have largely focused on conditions that affect cell turgor, i.e. induce osmotic stress. We show that chaotropic solutes that do not affect turgor reduce water activity, perturb macromolecule–water interactions and thereby destabilize cellular macromolecules, inhibit growth, and are powerful mediators of water stress in a typical soil bacterium, *Pseudomonas putida*. Chaotropic solute-induced water stress resulted mostly in the upregulation of proteins involved in stabilization of biological macromolecules and membrane structure. Many environmental pollutants and agricultural products are chaotropic chemicals and thus constitute a previously unrecognised but common form of biological stress in water bodies and soils.

Introduction

Water is life: water is the cytoplasmic milieu of cellular systems, is both a solvent and a reactant in biochemical processes, hydrates biological macromolecules and membranes, and provides lubrication, cohesion, hydraulic pressure, and cell turgor. Most of life in the biosphere depends ultimately upon photosynthesis in which water both facilitates the assimilation of CO_2 and functions as a key reactant. As a consequence, reduction in water availability constitutes a fundamental stress for cellular systems. Studies of water availability in biological systems have traditionally focused on osmotic stress, which influences cell turgor, which in turn influences fundamental processes such as transport, cell extension and growth, membrane integrity and regulation of cytosol concentration. However, other factors, such as chaotropic solutes that weaken electrostatic interactions in biological macromolecules, can influence water availability without having a major impact on cell turgor.

The goal of this work was to determine whether chaotropic solutes cause water stress in bacteria at concentrations at which they are likely to occur in the environment and, if so, how the bacteria respond. If solute effects on the structure and properties of water are responsible for their effects on macromolecule stability and water stress in cellular systems, then there may be a quantitative or semiquantitative relationship between these effects. In order to ascertain this, it was essential to quantify the effects of relevant concentrations of the selected compounds upon water activity, to quantify the effects of the chaotropes on the properties of a representative biological macromolecule (neither of which have, to our knowledge, been thus far determined for most chaotropic solutes), and to link these quantitative parameters to any stress responses detected. For this study, the cellular system we have chosen is *Pseudomonas putida* KT2440 (Bagdasarian *et al*., 1981; Timmis, 2002), a well-characterized model soil bacterium, which is a paradigm of the metabolically versatile Pseudomonads that are collectively able to metabolise a wide range of noxious organic compounds (Ramos *et al*., 1987; Timmis *et al*., 1994), many of which are chaotropes, and whose genome sequence was recently determined (Nelson *et al*., 2002). We show here that chaotropic compounds that do not affect turgor reduce water activity, perturb macromolecule–water interactions and thereby destabilize cellular macromolecules, inhibit growth, and are powerful mediators of water stress in *P. putida*. The bacterium responds by specifically upregulating the synthesis of proteins involved in stabilizing protein structure, in lipid metabolism, and in membrane composition.

Results

Quantification of water activity reduction induced by selected chaotropes

For this study, we selected seven chaotropic compounds – LiCl, ethanol, urea, ethylene glycol, phenol, guanidine

Received 17 January, 2003; revised 15 April, 2003; accepted 22 April, 2003. *For correspondence. E-mail jehalls@essex.ac.uk; Tel. (+44) 1206 872535; Fax (+44) 1206 872592.

Table 1. Characterization of solutes according to water activity reduction and chaotropic effect.[a]

Added compound (MW)	Water activity 1 M[b]	Concn. to cause 1°C decrease (mM)[c]	Chaotropic effect (kJ g^{-1} mole added compound^{-1})[d]	Concn. causing 50% growth inhibition (mM)[e]	Water activity at 50% growth inhibition[e]	Chaotropic effect at 50% growth inhibition (kJ g^{-1} solution)[e]
LiCl (42.40)	0.934	204	19.6	595	0.961	11.7
Ethanol (46.07)	0.967	700	5.92	600	0.980	3.54
Urea (60.06)	0.973	250	16.6	650	0.982	10.8
Ethylene glycol (62.07)	0.960	2500	1.66	1300	0.948	2.16
Phenol (94.11)	0.935[f]	29	143	9	0.999	1.29
Guanidine HCl (95.53)	0.943	130	31.9	125	0.993	3.98
Benzyl alcohol (108.13)	0.87	46	90.1	20	0.997	1.80
Heavy metals						
Sodium arsenate (185.98)	0.930[f]	40	105	0.806	1	0.0846
$HgCl_2$ (271.52)	0.75[f]	33	124	0.0027	1	0.000335

a. Chaotropic effect was calculated based on the arbitrary selection of a 1°C reduction in agar gel point.
b. Water activity values of 1 M solutions obtained using pure solutions of each compound, at 30°C (see *Experimental procedures* section).
c. Concentration of solute required to decrease gel point of 1.5% (w/v) agar solution by 1°C; values were derived from the gradients of the curves shown in Fig. 1.
d. The energy change to the agar-added compound solutions was calculated from the decrease in gel-point temperature that was caused by each solute. The molarity of each solute that is required to reduce gel point by 1°C was used to calculate the energy change per mole compound added, given that heat capacity for a 1.5% (w/v) agar gel is 4.15 kJ $g^{-1}°C^{-1}$ (slightly less than the heat capacity of water at 25°C which is approximately 4.20 kJ $g^{-1}°C^{-1}$; Cornillon *et al.*, 1995). The resulting values were an expression of the degree of chaotropicity of each compound.
e. When present in the medium of *P. putida*, grown at 30°C. Water activity values for these concentrations were obtained from curves drawn using a range of measured values.
f. The water activity values listed for 1 M solutions of phenol, benzyl alcohol and $HgCl_2$ were obtained by extrapolation of values obtained from more dilute solutions, due to the limited solubility of these compounds.

hydrochloride and benzyl alcohol – representing different chemical classes, molecular sizes and degrees of chaotropicity. As controls, we selected several other compounds and conditions – the heavy metals sodium arsenate, and $HgCl_2$, the antibiotic rifampicin and extremes of pH – that similarly inhibit bacterial growth without affecting water activity when applied at inhibitory levels.

The water activity values of aqueous solutions of chaotropic solutes, as well as heavy metals, were determined over a range of concentrations, at 30°C, by means of a Novasina IC II water activity instrument (Hallsworth and Nomura, 1999). Water activity values for 1 M solutions are listed in Table 1; due to the limited solubilities of phenol, sodium arsenate and $HgCl_2$, the values for these compounds were derived by extrapolation of values obtained for solutions of lower concentrations. The water activity values of the solutions measured were approximately proportional to the molecular weights of the corresponding solutes, with the lowest water activity values obtained for benzyl alcohol, sodium arsenate and $HgCl_2$.

Quantification of changes in macromolecule stability induced by chaotropes

Although physical parameters, such as water activity, vapour pressure, and water potential can be used to describe the water availability of solutions (Brown, 1990), they do not account for solute–macromolecule interactions, or exclusion of solutes from the aqueous phase of macromolecules (Arakawa and Timasheff, 1984; 1985). However, the structure and function of all cellular macromolecules are affected by the degree of chaotropicity or kosmotropicity (the tendency to order water, and strengthen electrostatic interactions in organic macromolecules) of solutes in their aqueous environments (Mansure *et al.*, 1994; Shah *et al.*, 1998). In particular, chaotropes affect hydrogen bonding and other electrostatic interactions of macromolecules. We have exploited the effect of such solutes on the gelling temperature of hydrophilic polymers as a convenient and direct means of quantifying solute chaotropic effects on macromolecules (see *Experimental procedures*). The gel-point temperature of agar was reduced by addition of each of the chaotropes listed above, as well as the heavy metals (Fig. 1; Table 1). Strongly chaotropic compounds (e.g. phenol) caused the greatest reduction of gel-point temperature, whereas the weakly chaotropic solute ethylene glycol caused only a slight decrease. The relative reductions in the gel-point temperature of gelatine by these compounds, were similar to those shown in Fig. 1 (data not shown). The slopes of the curves of Fig. 1 were used to calculate the amount of each solute required to decrease agar gel point by 1°C (Table 1), and the values obtained were used to calculate the chaotropic effect of each compound (Table 1; see also *Experimental procedures* section): phenol, benzyl alcohol and the heavy metals are seen to be strongly chaotropic at concentrations of 1 M, causing an energetic increase from 90.1 to 143 kJ g^{-1}, whereas the other compounds studied have more modest

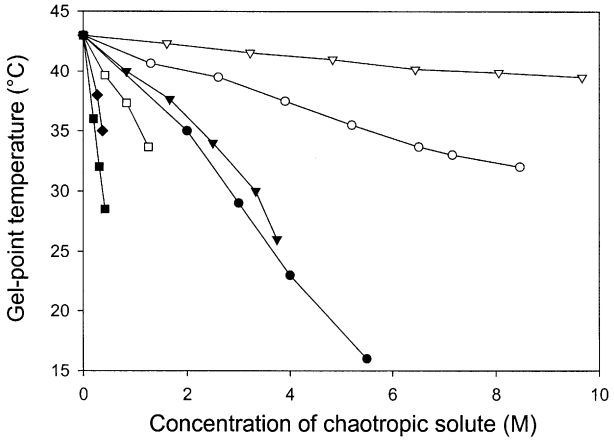

Fig. 1. Gel-point temperature of agar solutions (1.5% w/v at 30°C) containing chaotropic solutes over a range of concentrations: LiCl (●), ethanol (○), urea (▼), ethylene glycol (▽), phenol (■), guanidine hydrochloride (□) or benzyl alcohol (◆). The control solution that contained no added solute had a gel-point temperature of 43°C. All chaotropic solutes were incorporated into agar solutions to the maximum concentration possible without causing dissolution problems of the added compound or the agar.

effects, causing energetic increases ranging from 1.66 and 31.9 kJ g^{-1}. In contrast, virtually no reduction in gel point occurred as a result of varying pH over a wide range: between pH 2 and 11, in the case of agar (Fig. 2; see also *Experimental procedures* section), and between pH 4.5 and 10, in the case of gelatine (data not shown).

Thus, the reduction of the gel points of polysaccharide (agar) and protein (gelatine) macromolecules provides a biologically meaningful measure of chaotropicity of solutes, and a ranking of solutes according to their chaotropic effects (Table 1). This ranking is consistent with their molecular exclusion from/entrance into the aqueous phase of proteins and lipid bilayers (Arakawa and Timasheff, 1984; 1985; Carpenter and Crowe, 1988a), the ranking of ions in the Hofmeister series according to their effects on protein structure (Hofmeister, 1888), their effects on enzyme activity (e.g. Brown and Simpson, 1972; Millar et al., 1982), protein stabilization during cryopreservation or dehydration (Carpenter and Crowe, 1988b), their *in vivo* effects as denaturants in the case of chaotropes, or protectants in the case of kosmotropes (Arakawa and Timasheff, 1984; Hallsworth, 1998); the effect of solutes on membrane structure in liposomes and *in vivo* (Mansure *et al.*, 1994; Koynova *et al.*, 1997), and solute and temperature effects on nucleic acid structure (Rau and Parsegian, 1986; Saenger *et al.*, 1986; Marko and Siggia, 1994).

These results also explain a number of observations in stress biology, e.g. that chaotropes can compensate for the stress effects of kosmotropes or low temperature (Konopasek *et al.*, 2000), that kosmotropes can reduce the effects of chaotropes or high temperature (Mansure *et al.*, 1994; Taylor *et al.*, 1995), and that poor biodegradation of ethylparaben by *Pseudomonas cepacia* in the presence of methanol, propanol and benzyl alcohol (all chaotropic compounds) is partially compensated by the presence of Ca-alginate (Horiuchi *et al.*, 1994), a kosmotropic matrix.

Influence of chaotropes on growth of P. putida

Having demonstrated that the tested chaotropic solutes reduce water activity and macromolecule stability, we examined their ability to induce stress in a model cellular system, the bacterium *P. putida*, in terms of their effects on growth and genome expression. The heavy metals studied above, a classical growth inhibitor, rifampicin, and extremes of pH, were included as controls.

Plots of cell doubling times against concentrations of added solute are shown in Fig. 3. Interestingly, all plots of cultures exposed to chaotropic solutes, regardless of the chemical species, were initially characterized by plateaux. This indicates that growth rate was relatively unaffected by the concentration of the solute until a threshold concentration was reached, after which growth decreased sharply (Fig. 3A and B). In contrast, the growth rates of cultures containing heavy metals or rifampicin decreased progressively with solute concentration, as expected (Fig. 3C). The plots were used to determine the concentrations of solutes that produce a 50% inhibition in growth rate of a *P. putida* culture, and the increase in chaotropic energy in each case was calculated (Table 1) and used for the genome expression study carried out below.

The extremes of pH that were associated with a 50% decrease in the growth rate of *P. putida* were pH 5 and 9.6, values that result in no detectable effect on entropy or water relations in these two model systems (Fig. 2). At

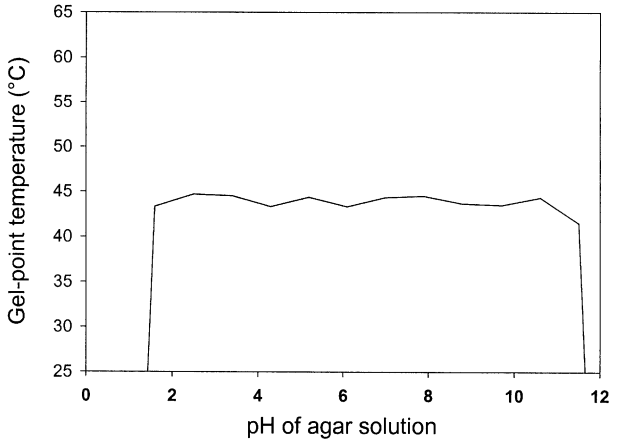

Fig. 2. Gel-point temperature of agar solutions (1.5%, w/v) over a range of pH; that had been modified by addition of concentrated HCl or 1 N NaOH.

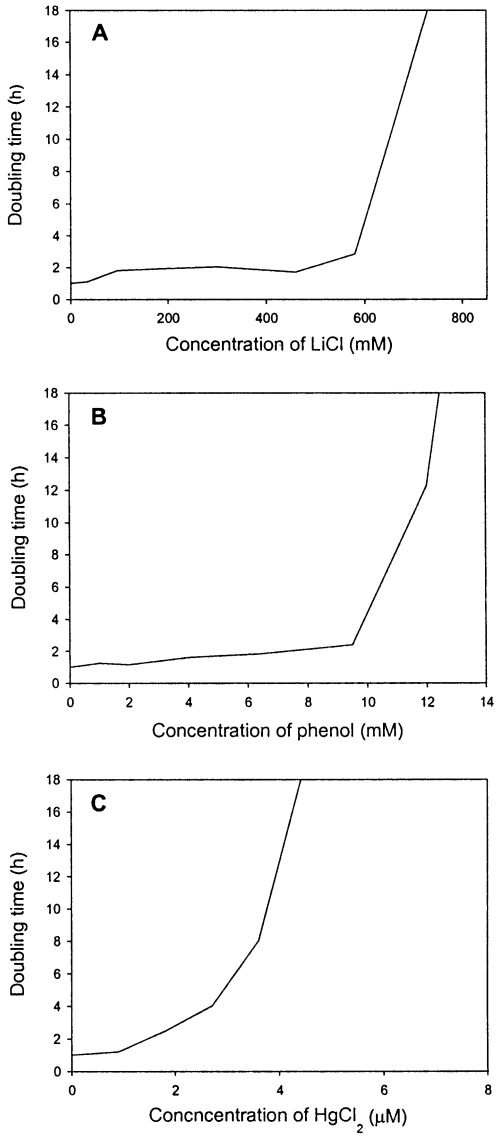

Fig. 3. Typical growth rate curves of *P. putida*, showing doubling time in the presence of (A) the chaotropic salt LiCl (B) the chaotropic aromatic solute phenol and (C) the heavy metal $HgCl_2$. All solutes were added over a concentration range. Treatments (A) and (B) are shown to illustrate the effect of chaotropic compounds on growth kinetics, compared with treatment (C); a heavy metal that illustrates a non-chaotropic treatment (see Table 1).

concentrations that reduced growth by 50%, the chaotropic effects of the chaotropic solutes ranged from 1.29 to 11.7 kJ g^{-1}, whereas those of the heavy metals were negligible (<0.1 kJ g^{-1}); the inhibitory effects of heavy metals are thus related to specific modes of toxicity, and not to chaotropicity.

Genomic expression responses to chaotropic solutes

The genetic responses of *P. putida* cells to chaotropic solutes was examined by genome expression profiling through proteomics, aided by the recent sequencing and annotation of the *P. putida* genome (Nelson *et al.*, 2002). In order to identify responses specific for conditions that increase entropy (i.e. chaotropic compounds and high temperature), inhibitory treatments that do not increase entropy, namely heavy metals, rifampicin and extremes of pH, were included in the experiment. Each treatment was designed to cause 50% inhibition in growth rate of the culture compared with that of a control culture without treatment. All conditions were maintained throughout the growth period of the cultures, i.e. the treatments were not administered as transient or shock effects.

The proteome study revealed 41 proteins that were up- or downregulated by the treatments imposed; these proteins are listed in Table 2 according to their principal metabolic functions. Treatments that increased entropy (Table 1) caused the upregulation of many more proteins (Thome and Muller, 1991) than other inhibitory treatments (three), whereas they caused the downregulation of 13 proteins, approximately the same number downregulated by other treatments. Three proteins (HtpG, DnaK, GroEL) involved in the stabilization of proteins under thermodynamically unfavourable conditions were upregulated by chaotropic solutes and high temperature, and another two (IbpA and trigger protein) by chaotropic solutes alone, whereas four of these plus one other (SlyD) were downregulated by inhibitory treatments that do not affect entropy (Table 2, e.g. see Fig. 4).

Two proteins involved in lipid metabolism (FabB, FadBlx) and one outer membrane protein (H1) were upregulated, whereas two other outer membrane proteins (D and F) were downregulated in these treatments. In contrast, inhibitory treatments that do not increase entropy had little detectable effect on the expression of proteins involved in lipid metabolism or membrane structure. Outer membrane protein H1 of *Escherichia coli* is involved in protein translocation across the plasma membrane and/or sorting to the outer membrane, and acts as a folding catalyst or chaperone (Thome and Muller, 1991; Missiakas *et al.*, 1996). An absence of outer membrane protein F is associated with reduced membrane permeability (Angus *et al.*, 1982; Yoshimura and Nikaido, 1982) and toluene tolerance (Li *et al.*, 1995) in *Pseudomonas aeuginosa*. It is well established that chaotropic compounds and high temperature reduce membrane lipid order and induce changes in membrane composition (Heipieper *et al.*, 1992; Slater *et al.*, 1993; Nobre *et al.*, 1996; Brown *et al.*, 2000). The observed increase in protein H1 in cells grown in the presence of chaotropic solutes, and the decrease in protein F in cells exposed to conditions that increase entropy (i.e. high temperature or chaotropic solutes; Table 1) are consistent with the known properties of these outer membrane proteins.

Table 2. Proteins from *P. putida* cells grown under inhibitory conditions that did, or did not, exert a chaotropic effect.[a]

Protein name and function[b]	Decreased[c]		Increased[c]	
	Chaotropes and temperature[d]	Other inhibitory treatments[e]	Chaotropes and temperature[d]	Other inhibitory treatments[e]
Protein stabilization				
Heat shock protein 90 (HtpG)	L, E, P, G, B, T	_	_	S, M, R, 5, 9.6
Heat shock protein 70 (DnaK)	E, P, B, T	_	_	S, M, 5
Heat shock protein 60 (GroEL)	E, P, B, T	_	_	S, R, 5
Heat shock protein A (IbpA)	P, B	_	_	_
Trigger protein	E, P, B	_	T	S, M, R, 5
Peptidyl-propyl *cis-trans*	_	_	_	S
Isomerase (SlyD)	_	_	_	M, R, 5, 9.6
Cell envelope/lipid metabolism				
3-oxoacyl-acyl carrier protein synthase I (FabB)	L, E, U, EG, P, G, B	_	_	_
Putrescine transporter binding protein (Pot f)	T	_	_	_
Enoyl-CoA hydratase/Isomerase (FadBlx)	E, P, B	_	_	_
Outer membrane protein D (OmpD)	_	_	P, B, T	_
Outer membrane protein F (OmpF)	_	_	L, EG, P, B, T	_
Outer membrane protein G (OmpG)	_	_	T	_
Outer membrane protein H1 (OmpH 1)	L, E, U, G	5	_	R
GDP-mannose dehydrogenase (Gmd)	T	_	_	_
Detoxification				
Catalase/peroxidase	P, B	_	_	_
Superoxide dismutase (SodB)	E	_	_	R
Peroxidase	E	_	_	_
Xenobiotic reductase A (XenA)	T	_	_	_
Alkyl hydroperoxide reductase (AhpC)	_	_	L, EG, T	R
Protein synthesis				
30S ribosomal protein S1 (RpsA)	L, E, U, P, G, B, T	_	_	S, M, 5
Translation elongation factor (EF-TU)	T	_	_	5
Translation elongation factor G (FusA)	E, P, B	_	_	_
Translation elongation factor P	_	_	T	_
Alanyl-t-RNA synthetase (Alas)	_	_	L, E, U, EG, P, G, B	_
Energy metabolism				
Aconitate hydratase (AcnB)	E, P, G, B	_	_	_
D-β hydroxybutyrate dehydrogenase (BdhA)	P, B	_	_	_
CoA-hydrolase/transferase family protein	E	_	_	_
ATP synthase delta chain	_	_	P, B, T	_
Succinyl CoA sythetase alpha subunit	_	_	T	_
Central intermediary metabolism				
Inorganic pyrophosphatase (Ppa)	_	_	_	S, M, 5, 9.6
Hydrolase Orf 07396	_	_	_	S, M, 5
Other				
Heat shock protein M (ClpB-protein)	T	_	_	_
Ferrisiperphore receptor-like protein; Orf 02803	T	_	_	_
GTP-binding protein (GTP1); Orf 07432	T	_	_	_
Flagellin (FliC)	_	_	L, E, U, P, G, B, T	M, 9.6
Chorismate mutase (TyrA)	_	_	T	_
Nicotinate-nucleotide pyrophosphorylase (NadC); Orf 07273	_	_	T	_
DNA-directed RNA-polymerase alpha subunit (RpoA)	_	_	T	S
Conserved hypothetical protein; Orf 00749	_	M	_	_
Conserved hypothetical protei Orf 00938	P, B	S	_	_
Conserved hypothetical protein; Orf 03678	P, B, T	_	_	_

a. See Table 1; all treatments inhibited growth rate by 50% when compared with control cultures. Chaotropic solutes and high temperature affected water activity and increased chaotropicity, whereas heavy metals, rifampicin, extremes of pH did not. L = LiCl, E = ethanol, U = urea, EG = ethylene glycol, P = phenol, G = guanidine hydrochloride, B = benzyl alcohol, T = 44°C, S = sodium arsenate, M = $HgCl_2$, R = rifampicin, 5 = pH 5, and 9.6 = pH 9.6 treatment.
b. Proteins are grouped according to their main metabolic function.
c. Significant increases and decreases in protein concentration relative to those from control cells.
d. i.e. LiCl, ethanol, urea, ethylene glycol, phenol, guanidine hydrochloride, benzyl alcohol (see Table 1) and high temperature (44°C).
e. i.e. Heavy metals (see Table 1), rifampicin (4.5 nM), and extremes of pH (pH 5 and 9.6).

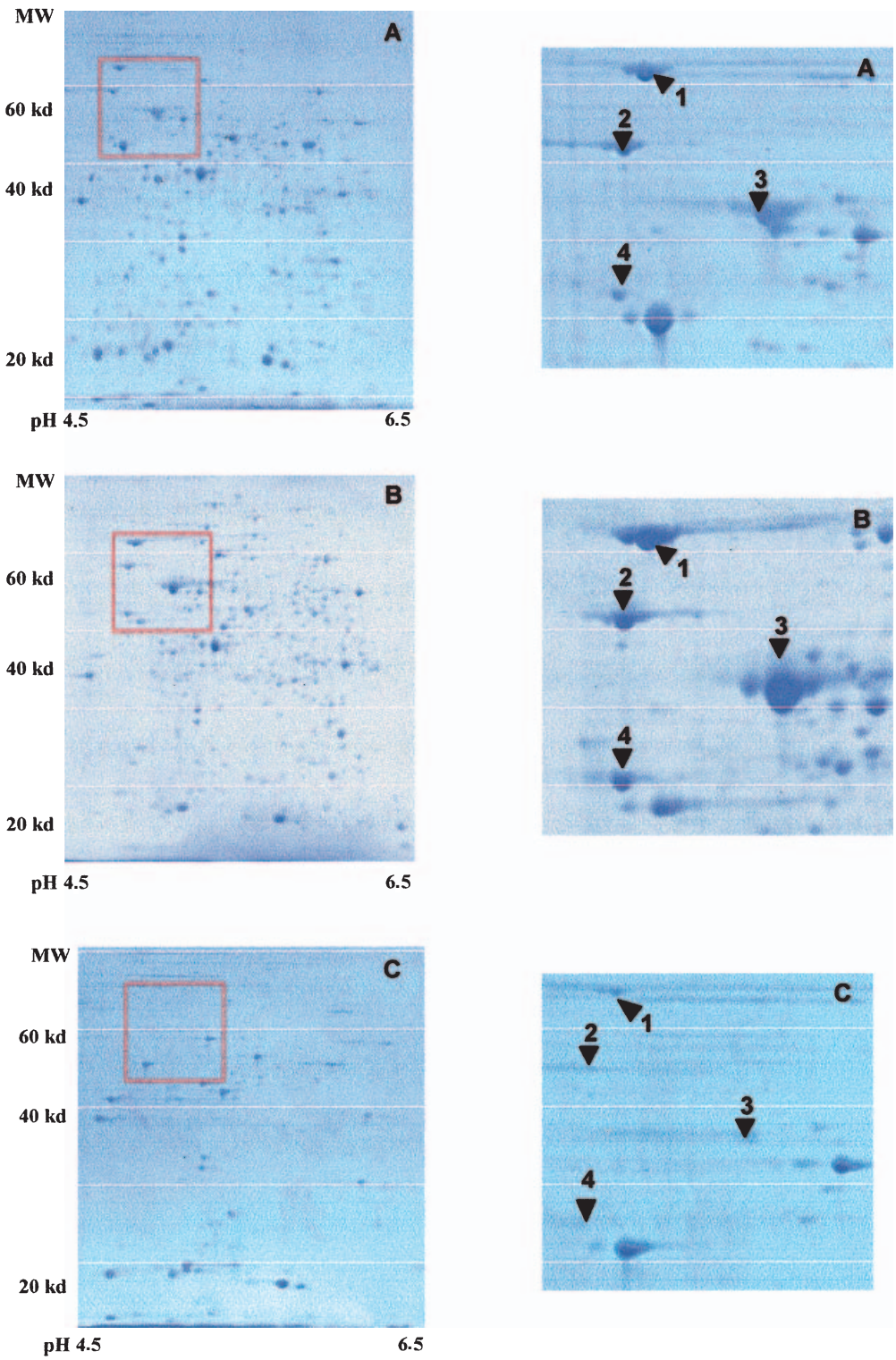

Fig. 4. Two-dimensional gels of the proteome of *P. putida* cells in a standard medium, subjected to chaotrope-induced water stress, and subjected to a specific inhibitor. The gels show the major proteins of *P. putida* cells during exponential growth at 30°C in liquid medium containing (A) no added solute (control) (B) 9 mM phenol (chaotropic inhibitor) and (C) 806 μM sodium arsenate (non-chaotropic inhibitor). The growth rate of cells in the phenol and sodium arsenate treatments was 50% lower than that of the control culture. Each gel was loaded with proteins extracted from an equivalent number of cells. The labelled proteins in the inserts are protein-stabilizing proteins: 1: heat shock protein 90 (HtpG), 2: heat shock protein 70 (DnaK), 3: heat shock protein 60 (GroEL) and 4: trigger protein.

Two proteins involved in protein synthesis (RpsA and FusA) were upregulated by several conditions that increase entropy (Table 1), whereas one (AlaS) was downregulated. By contrast, the S1 ribosomal protein RpsA was downregulated by heavy metals and low pH. In general, there were more proteins visible in the gels of bacteria treated with phenol than in gels of bacteria treated with sodium arsenate, which is consistent with the upregulation of proteins involved in protein synthesis by chaotropic compounds and their downregulation by non-chaotropic compounds or treatments (Fig. 4; Table 2; see also Botsford, 1990; Heyde and Portalier, 1990).

Several proteins, such as catalase, peroxidase, superoxide dismutase, involved in elimination of active oxygen species, were upregulated by the chaotropic solutes ethanol, phenol and benzyl alcohol, and high temperature, whereas the latter protein was downregulated by the rifampicin treatment. Three proteins involved in energy metabolism (AcnB, Bdh, a CoA-hydrolase/transferase family protein) were upregulated, and one other (ATP synthase delta chain) was downregulated, under several conditions that increase entropy. Flagellin (FliC) was downregulated in response to most of the treatments, including all that increase entropy, and by $HgCl_2$ and pH 9.6, which suggests that growth inhibition caused by diverse effects may reduce motility. Regulatory interactions between stress responses and flagellum biosynthesis have been established in several microbial species (Haller and Fabry, 1998; Han *et al*., 1999). Two conserved hypothetical proteins were also upregulated by phenol and benzyl alcohol, one of which was also upregulated by growth at 44°C.

Discussion

Soils are habitats that are often subjected to major, climate-mediated oscillations in water content and activity and, in addition, may receive substantial inputs of natural and anthropogenic solutes as a result of agricultural and industrial activities. Soil microorganisms play key roles in element cycling, maintenance of soil fertility, nutrient supply to plants, inhibition of fungal pathogens, etc. Water stress is a constant challenge for soil microorganisms, and their ability to sense, respond and adapt to water stress, is vital to their metabolic functions, growth and survival.

Saprophytic microbes are responsible for detoxifying and mineralising a multitude of chemicals that enter the biosphere and are essential to the health of our environment. Although metabolism of such compounds, and their specific toxic effects on biological systems, have been studied extensively, their possible activities as agents of water stress on the organisms involved in their metabolism have not so far been studied. In addition to the inherent importance of gaining an understanding of non-osmotic, chemical-induced water stress in biological systems, this subject has important implications for biological waste treatment and bioremediation – the biotechnological exploitation of microorganisms to accelerate the removal of toxic chemicals from the environment.

In this study we have demonstrated that chaotropic compounds that freely traverse biological membranes, and hence do not affect turgor (Guijarro and Lagunas, 1984; Giocondi and Legrimellec, 1991; Kiyosawa, 1991; Shah *et al*., 1998; Takahashi *et al*., 2000), are powerful mediators of water stress. Indeed, it may be that many so-called toxic compounds have negligible specific modes of action on specific cellular targets, but rather are agents of water stress with general adverse effects on cellular macromolecules (Hallsworth, 1998).

Pseudomonas putida, a typical soil bacterium that is continuously exposed, and hence presumably well adapted to climatically and environmentally influenced variations in water availability, as well as chaotropic environmental pollutants, responded to exposure to chaotropic compounds by upregulating proteins that mediate protein stabilization, or are involved in lipid metabolism, membrane structure, protein synthesis and energy metabolism. This is consistent with a general response to counteract water-related perturbations in macromolecular structure and function, and in cellular organelles, through an increase in the levels of cellular functions involved in protein and membrane stability and an associated requirement for an increase in protein synthesis and energy generation (Ramos *et al*., 1987; Benndorf *et al*., 1999; Brown *et al*., 2000; Park *et al*., 2001). Cellular responses and adaptations to stress have a diagnostic value by indicating or confirming changes in the structure and other properties of water, that can affect water: macromolecule interactions a and thereby cause entropy-related stress (Hallsworth and Magan, 1994; 1995; Hallsworth, 1998). Depending upon the type of cell, metabolic changes that counter adverse thermodynamic conditions may include: production of compatible solutes, protectants, late embryogenic proteins, heat shock proteins, chaperones, membrane lipids (e.g. different chain length, degree of branching or polar head group), and/or sterols.

Pseudomonas putida cells exposed to chaotropes did not experience growth inhibition until critical solute concentrations were reached, whereas those subjected to other inhibitory treatments not inducing a water stress response experienced increasing growth inhibition with increasing treatment level. This suggests that *P. putida* cells are able to adapt their physiology to chaotrope perturbation, by means of the water stress response, but less readily to specific chemical intoxication or other specific non-chaotrope forms of stress. In other words, destabili-

zation of macromolecules is a more elastic process than specific inactivation, and can be functionally reversed up to a critical thermodynamic point. If, indeed, solutes that increase entropy and affect hydration of macromolecules are characterized by fundamentally different dose-growth rate responses than those which cause specific toxic effects, then dose-growth rate responses may be a useful predictor of the principal cause of metabolic inhibition by chemical agents.

Metabolically versatile bacteria like *Pseudomonas* that can metabolise natural and xenobiotic pollutants play a major role in eliminating such pollutants from the environment, and hence in maintaining environmental quality. They are therefore exploited in bioremediation, biotechnological processes to clean up contaminated soils and wastewaters. Though environmentally friendly and relatively inexpensive, both the natural attenuation of pollutants, and bioremediation, can be slow processes (Smets *et al*., 2002), a key factor that restricts the range of bioremediation applications. Efforts to achieve higher degradation rates often founder on a lack of understanding of the rate-limiting factors, and of potential ways and means of circumventing them. Water stress has not so far been studied in detail in soil organisms important for biodegradation, but this report suggests that chaotrope-induced water stress will affect their metabolic activities. As many environmental pollutants are chaotropes, this finding has implications for the rate of degradation of xenobiotic compounds, and suggests that genetic interventions to optimise resistance and adaptation to water stress may be beneficial to such processes, particularly those involving bioaugmentation of soils by addition of specific biodegrading species.

Experimental procedures

Determination of water activity

The water activities of solutions of each chaotropic solute were determined using a Novasina IC II water activity machine fitted an alcohol-resistant humidity sensor and eVALC alcohol filter (Novasina, Pfäffikon, Switzerland) as described previously (Hallsworth and Nomura, 1999), over a range of concentrations at 30°C. This equipment was calibrated using saturated salt solutions of known water activity (Winston and Bates, 1960). For comparison, the water activities of sodium arsenate and $HgCl_2$ solutions were also determined. Values were determined three times using replicate solutions made up on separate occasions. The variation of replicate values was within ± 0.002 a_w.

Agar and gelatine gel-point determination

Extra Pure Reagent grade agar was obtained from Nacalai Tesque, Kyoto, Japan (gel strength; 600–700 g cm^2) and 300 bloom gelatine was obtained from Sigma, USA. Erlenmeyer flasks (250 ml) containing 1.5 g agar or 1.6 g gelatine and made up to 40 ml with distilled water were sealed with bungs of non-absorbent cotton wool and placed in a water bath at 95°C to dissolve the agar, or 45°C to dissolve the gelatine. Solutions of the compounds under study were added at different concentrations to give a final volume of 100 ml at 25°C. The agar solutions were allowed to cool to about 55°C before addition the chaotropic compounds. Before the addition of chaotropic compounds to the agar or gelatine, the solutions were heated to aid dissolution if necessary. For each compound a range of concentrations was studied, up to the maximum concentration possible without encountering precipitation of the compound or the agar or gelatine.

The agar- and gelatine-compound solutions were allowed to cool gradually and the gel-point temperature was recorded using a temperature probe (Jenway, UK). The gel transition was sudden, and was detectable by eye, resulting in temperature measurements that were reproducible to within ±0.3°C. Upon gelation, the pH of all agar- and gelatine-compound solutions were measured using Acilit indicator strips (Merck) and was found to be within the range 5–8. The effect of pH on gel point of agar and gelatine was assessed within the range of pH 1 and pH 12, at intervals of 0.5, by adjustment with concentrated HCl or 1 N NaOH. The data obtained were used to calculate the chaotropic effect of each compound in kJ g^{-1} mole added per compound, and for the concentration of each solute that caused 50% growth inhibition of *P. putida* (Table 1). The values were calculated based on the knowledge that the heat capacity for a 1.5% (w/v) agar gel is 4.15 kJ $g^{-1}°C^{-1}$ (slightly less than the heat capacity of water at 25°C which is approximately 4.20 kJ $g^{-1}°C^{-1}$; see Cornillon *et al*., 1995).

Organism and growth conditions

Pseudomonas putida KT2440, obtained from the DSMZ, Braunschweig, was cultivated in modified Luria–Bertani broth (10 g yeast extract (Difco) and 5 g Bacto peptone (Difco) made up to 1 l with deionized water) or Luria–Bertani agar (containing an additional 15 g agar l^{-1}). For growth and proteomic studies, liquid cultures were incubated on rotary shaking platforms at 160 r.p.m. at a temperature of 30°C, unless stated otherwise. Chaotropic solutes, as well as heavy metals and the antibiotic rifampicin, were added after media had been autoclaved and immediately before inoculation with *P. putida*. Rifampicin was added from a stock solution in methanol (300 mg ml^{-1}). The water activity of culture media was 0.001 lower than the values obtained from solution measurements, due to the water activity reduction caused by the yeast extract and peptone in the medium. The water activity of culture media was also determined during the late exponential phase of growth, to ensure that the water activity had not changed during growth of the cultures: no changes of water activity that exceeded the limits of sensitivity of the Novasina IC II water activity machine were detected for any media.

Growth assessment and growth rate determination

Conical flasks (300 ml) containing 100 ml of medium were inoculated with 0.5 ml of cell suspension (final turbidity at 560 nm below 0.1) taken from an exponentially growing cul-

ture of *P. putida*, incubated, and samples taken at hourly intervals to obtain growth curves. Turbidity values were converted to cell number using a standard curve, and the doubling times during the exponential growth phase were calculated from the slopes of the growth curves. These data were used to establish the relationship between solute concentration and growth rate.

Cell harvesting and protein extraction

Cultures were harvested during exponential growth, once the turbidity had reached 0.54–0.6, by centrifugation at 4°C at 10 000 *g* for 15 min, resuspended in phosphate-buffered saline (PBS: 10 mM sodium phosphate buffer, pH 7.4, 138 mM NaCl; Sambrook *et al*., 1989). Harvested cells were washed four times in PBS, and then resuspended in PBS buffer, the suspensions were aliquoted in microfuge tubes and pelleted by centrifugation, such that all pellets contained cell biomass corresponding to approximately 16 ml of the original culture. The pellet-containing microfuge tubes were stored at −20°C overnight.

For protein extraction, a cell pellet was allowed to thaw on ice, 1.25 ml reswelling solution (4% w/v 3-(3-cholamidopropyl)-di-methylammonio-1-propane-sulfonate (CHAPS), 30 mM dithiothreitol (DTT), 20 mM TRIS base, 7.5 M urea, 2 M thiourea, and one tablet of protease inhibitor cocktail (Complete™ Mini, Boehringer, 20 ml^{-1}) was added, and the suspension sonicated with a 3.5-mm sonication probe (Labsonic U; Braun) six times using 30 s pulses, with 30 s intervals in between each. The microfuge tubes were allowed to stand at room temperature for 15 min and then centrifuged to remove cell debris. In order to remove salts, DNA and other contaminating substances from the extracts, 4.8 ml of methanol–chloroform (3:1) were added to each sample and the suspension mixed by vortexing, 3.6 ml of distilled water was added, the sample was vortexed for 1 min, before centrifugation for 5 min at 10 000 *g*. The upper phase was discarded, and then 3.6 ml of methanol were added and the suspensions mixed by vortexing, followed by centrifugation for 5 min. The protein pellets were air dried, resuspended in 300 µl reswelling solution, and the protein concentration determined according to Bradford (1976).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out according to Görg *et al*., 1991). From each microfuge tube, 280 µl was applied by reswelling to 4–7 IPG ReadyStrips (Bio-Rad) overnight. Each gel was loaded with proteins extracted from an equivalent cell biomass. Isoelectric focusing was performed in a Multiphor (Amersham Pharmacia Biotech) at a maximum voltage of 3500 V for 200 kVh in total. The strips were then transferred to 1.5 mm-thick gradient sodium dodecyl sulphate (12%, w/v)– polyacrylamide (15%, w/v) gels, and developed using the IsoDalt system (Amersahm Pharmacia Biotech) overnight. The gels were stained using colloidal Coomassie R-250.

Identification of proteins

Proteins were identified by mass spectrometry using peptide fingerprints. Protein spots were cut out of gels, destained and prepared for matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) analysis, according to Wissing *et al*. (2000). The peptide mass fingerprints obtained were identified using a *P. putida* (KT2440) protein database that was constructed in conjunction with a genome sequencing study (Nelson *et al*., 2002).

Acknowledgements

We are indebted to Professor P. Nicholls, Dr T. J. McGenity and Professor M. T. Wilson (University of Essex, UK), Professor A. R. Cossins (University of Liverpool) and Dr C. Heintz for their advice. Experimental assistance was kindly provided by D. Bludau and D. Regenhardt (GBF – National Research Centre for Biotechnology, Germany). The work was supported by the EU (MIFRIEND) and BMBF (German Ministry of Education, Science, Research and Technology).

References

Angus, B.L., Carey, A.M., Caron, D.A., Kropinski, A.M.B., and Hancock, R.E.W. (1982) Outer membrane permeability in *Pseudomonas aeruginosa* – comparison of a wild-type with an antibiotic susceptible mutant. *Antimicrob Agents* **21:** 299–309.

Arakawa, T., and Timasheff, S.N. (1984) Mechanism of protein salting in and salting out by divalent-cation salts – balance between hydration and salt binding. *Biochem* **23:** 5912–5923.

Arakawa, T., and Timasheff, S.N. (1985) The stablization of proteins by osmolytes. *Biophys J* **47:** 411–414.

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N. (1981) Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16:** 237–247.

Benndorf, D., Loffhagen, N. and Babel, W. (1999) Induction of heat shock proteins in response to primary alcohols in *Acinetobacter calcoaceticus*. *Electrophoresis* **20:** 781–789 1999.

Botsford, J.L. (1990) Analysis of protein expression in response to osmotic-stress in *Escherichia coli*. *FEMS Microbiol Lett* **72:** 355–360.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Brown, A.D. (1990) Microbial water stress physiology – principles and perspectives. Chichester: Wiley pp. 2–14.

Brown, A.D., and Simpson, J.R. (1972) Water relations of sugar-tolerant yeasts: the role of intracellular polyols. *J Gen Microbiol* **72:** 589–591.

Brown, G.R., Sutcliffe, I.C., Bendell, D., and Cummings, S.P. (2000) The modification of the membrane of *Oceanomonas baumannii* (T) when subjected to both osmotic and organic solvent stress. *FEMS Microbiol Lett* **189:** 149–154.

Carpenter, J.F., and Crowe, J.H. (1988a) Modes of stabilization of a protein by organic solutes during desiccation. *Cryobiology* **25:** 459–470.

Carpenter, J.F., and Crowe, J.H. (1988b) Fundamental differences in the interactions of stabilizing solutes with proteins during freeze-thawing versus during freeze-drying. *Cryobiology* **25:** 537–538.

Cornillon, P., Andrieu, J., Duplan, J.C., and Laurent, M. (1995) Use of nuclear-magnetic-resonance to model thermophysical properties of frozen and unfrozen model food gels. *J Food Eng* **25:** 1–19.

Giocondi, M.C., and Legrimellec, C. (1991) Water permeation in Madin-Darby canine kidney-cells is modulated by membrane fluidity. *Biochim Biophys Acta* **1064:** 315–320.

Görg, A. Postel, W., Friedrich, C., Kuick, R., Strahler, J.R., and Hanash, S.M. (1991) Temperature-dependent spot positional variability in 2-dimensional polypeptide patterns. *Electrophoresis* **12:** 653–658.

Guijarro, J.M., and Lagunas, R. (1984) *Saccharomyces cerevisiae* does not accumulate ethanol against a concentration gradient. *J Bacteriol* **160:** 874–878.

Haller, K., and Fabry, S. (1998) Brefeldin A affects synthesis and integrity of a eukaryotic flagellum. *Biochem Biophys Res Comm* **242:** 597–601.

Hallsworth, J.E. (1998) Ethanol-induced water stress in yeast. *J Ferment Bioeng* **85:** 125–137.

Hallsworth, J.E., and Magan, N. (1994) Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. *Microbiol SGM* **140:** 2705–2713.

Hallsworth, J.E., and Magan, N. (1995) Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiol SGM* **141:** 1109–1115.

Hallsworth, J.E., and Nomura, Y. (1999) A simple method to determine the water activity of ethanol-containing samples. *Biotechnol Bioeng* **62:** 242–245.

Han, Y.P.W., Uhl, M.A., Han, S.J., and Shi, W.Y. (1999) Expression of bvgAS of. *Bordetella pertussis* represses flagellar biosynthesis of *Escherichia coli*. *Arch Microbiol* **171:** 127–130.

Heipieper, H.J., Diefenbach, R., and Keweloh, H. (1992) Conversion of *cis* unsaturated fatty-acids to trans, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl Environ Microb* **58:** 1847–1852.

Heyde, M., and Portalier, R. (1990) Acid shock proteins of *Escherichia coli*. *FEMS Microbiol Lett* **69:** 19–26.

Hofmeister, F. (1888) On the understanding of the effects of salts. *Arch Exp Pathol Pharmakol* **24:** 247–260.

Horiuchi, K., Morimoto, K., Kobashi, K., Mitsu, T., and Suemitsu, R. (1994) Transesterification of ethylparaben by free and entrapped cells of *Pseudomonas cepacia*. *J Ferment Bioeng* **77:** 324–325.

Kiyosawa, K. (1991) Volume tric properties of polyols (ethylene-glycol, glycerol, meso-erythritol, xylitol and mannitol) in relation to their membrane-permeability – group additivity and estimation of the maximum radius of their molecules. *Biochim Biophys Acta* **1064:** 251–255.

Konopasek, T., Strzalka, K., and Svobodova, J. (2000) Cold shock in *Bacillus subtilis* different effects of benzyl alcohol and ethanol on the membrane. *BBA biomembranes* **1464:** 18–26.

Koynova, R., Brankov, J., and Tenchov, B. (1997) Modulation of lipid phase behavior by kosmotropic and chaotropic solutes – Experiment and thermodynamic theory. *Eur Biophys J Biophys Lett* **25:** 261–274.

Li, L., Komatsu, T., Inoue, A., and Horikoshi, K. (1995) A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. *Biosci Biotechn Bioch* **59:** 2358–2359.

Mansure, J.J.C., Panek, A.D., Crowe, L.M., and Crowe, J.H. (1994) Trehalose inhibits ethanol effects on intact yeast-cells and liposomes. *BBA Biomembranes* **1191:** 309–316.

Marko, J.F., and Siggia, E.D. (1994) Fluctuations and supercoiling of DNA. *Science* **265:** 506–508.

Millar, D.G., Griffiths-Smith, K., Algar, E., and Scopes, R.K. (1982) Activity and stability of glycolytic enzymes in the presence of ethanol. *Biotechnol Lett* **4:** 601–606.

Missiakas, D., Schwager, F., Betton, J.M., Georgopoulos, C., and Raina, S. (1996) Identification and characterization of HsIV HsIU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in *Escherchia coli*. *EMBO J* **15:** 6899–6909.

Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins Dos. Santos, V., *et al.* (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4:** 799–801.

Nobre, M.F., Carreto, L., Wait, R., Tenreiro, S., Fernandes, O., da Sharp, R.J., and Costa, M.S. (1996) Fatty acid composition of the species of the genera *Thermus* and *Meiothermus*. *Systemat Appl Microbiol* **19:** 303–311.

Park, S.H., Oh, K.H., and Kim, C.K. (2001) Adaptive and cross-protective responses of *Pseudomonas* so DJ-12 to several aromatic and other stress shocks. *Curr Microbiol* **43:** 176–181.

Ramos, J.L., Wasserfallen, A., Rose, K., and Timmis, K.N. (1987) Redesigning metabolic routes – manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. *Science* **235:** 593–596.

Rau, D.C., and Parsegian, V.A. (1986) Dissection of the solvent entropy change driving molecular assembly – chaotropic and lyotropic anions in the Mn-induced condensation of DNA. *Biophys J* **49:** A301 Part 2.

Saenger, W., Hunter, W.N., and Kennard, O. (1986) DNA conformation is determined by economics in the hydration of phosphate groups. *Nature* **324:** 385–388.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning*. Cold Spring Harbor, New York: Cold Spring Harbor Press

Shah, D., Johnston, T.P., and Mitra, A.K. (1998) Thermodynamic parameters associated with guanidine HCl- and temperature-induced unfolding of bFGF. *Int J Pharm* **169:** 1–14.

Slater, S.J., Ho, C., Taddeo, F.J., Kelly, M.B., and Stubbs, C.D. (1993) Contribution of hydrogen-bonding to lipid lipid interactions in membranes and the role of lipid order – effects of cholesterol, increased phospholipid unsaturation, and ethanol. *Biochem* **32:** 3714–3721.

Smets, B.F., Siciliano, S.D., and Verstraete, W. (2002) Natural attenuation: extant microbial activity forever and ever? *Environ Microbiol* **4:** 315–317.

Takahashi, H., Matsuo, A., and Hatta, I. (2000) Effects of chaotropic and kosmotropic solutes on the structure of lipid

cubic phase: Monoolein-water systems. *Mol Crystals Liquid Crystals* **347:** 475–482.

Taylor, L.S., York, P., Williams, A.C., Edwards, H.G.M., Mehta, V., Jackson, G.S., *et al.* (1995) Sucrose reduces the efficiency of protein denaturation by a chaotropic agent. *BBA Protein Struct M* **1253:** 39–46.

Thome, B.M., and Muller, M. (1991) Skp is a periplasmic *Escherchia coli* protein requiring SecA and SecY for export. *Mol Microbiol* **5:** 2815–2821.

Timmis, K.N. (2002) Pseudomonas putida*: a cosmopolitan opportunist* par excellence. *Environ Microbiol* **4:** 779–781.

Timmis, K.N., Steffan, R.J., and Unterman, R. (1994) Designing microorganisms for the treatment of toxic wastes. *Annu Rev Microbiol* **48:** 525–557.

Winston, P.W., and Bates, D.H. (1960) Saturated solutions for the control of humidity in biological research. *Ecology* **41:** 232–237.

Wissing, J., Heim, S., Flohe, L., Bilitewski, U., and Frank, R. (2000) Enrichment of hydrophobic proteins via Triton X-114 phase partitioning and hydroxyapatite column chromatography for mass spectrometry. *Electrophoresis* **21:** 2589–2593.

Yoshimura, F., and Nikaido, H. (1982) Permeability of *Pseudomonas aeruginosa* outer-membrane to hydrophilic solutes. *J Bacteriol* **152:** 636–642.