Evidence for the existence of a novel component of biological water stress (anhydrotic stress) in *Escherichia coli*

G. UMAPRASAD and J. GOWRISHANKAR*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Abstract

The pathways for biosynthesis of pyrimidines, L-arginine and the polyamines are intimately interrelated in many microorganisms. We discovered in this study that growth of wild-type *Escherichia coli* in low-water-activity minimal media is inhibited by the addition of uracil. Uracil sensitivity was observed irrespective of whether the dissolved solute(s) contributing to decreased water activity was ionic (e.g. NaCl, K_2SO_4), nonionic and impermeable (e.g. sucrose), nonionic and freely permeable (e.g. glycerol), or any mixture of these types. A mutant resistant to such growth inhibition was isolated and was shown to harbour a bradytrophic mutation in *argA*, the gene encoding the first step in the L-arginine biosynthetic pathway. Mutations in *argR*, whose product is the aporepressor of the same pathway, or exogenous supplementation with L-arginine or L-citrulline, also conferred resistance to uracil inhibition in low-water-activity media. A similar uracil-sensitivity phenotype, which was reversible by *argA*, *argR*, or L-arginine addition, was exhibited even in media with a more moderate reduction in water activity in two different situations: for a *speC* mutant (which is defective in the enzyme ornithine decarboxylase required for biosynthesis of the polyamines) and for the wild-type strain in media additionally supplemented with L-ornithine. On the basis of these observations, we propose a model in which high cytoplasmic levels of the intermediary metabolite L-ornithine are inhibitory to growth of *E. coli* in media of low water activity. Our results also provide the first evidence for the existence of a third component of physiological water stress, which is elicited by both impermeable and permeable dissolved solutes (the other two known components are ionic stress, which is elicited only by ionic solutes, and osmotic stress, which is elicited only by impermeable solutes either ionic or nonionic). We propose the term anhydrotic stress to refer to this novel component of water stress.

[UmaPrasad G. and Gowrishankar J. 1998 Evidence for the existence of a novel component of biological water stress (anhydrotic stress) in *Escherichia coli*. *J. Genet.* **77**, 1–11]

Introduction

Water stress imposed by drought, desiccation, dissolved solutes or ice formation is an abiotic stress encountered by a variety of organisms including microbes, plants and aquatic animals. The cellular mechanisms for adaptation to water stress appear to be remarkably similar in the diverse biological kingdoms (Yancey *et al.* 1982; Le Rudulier *et al.* 1984; Csonka 1989; Csonka and Epstein 1996).

The mechanism of inhibition of growth of biota by a dissolved solute such as NaCl can be subdivided into two components (Greenway and Munns 1980; Wyn Jones 1984; Csonka and Epstein 1996). The first component is referred to as salinity stress or ionic stress, and is specific to the chemical species of ions in solution; adaptation to ionic stress is often achieved by processes that exclude the toxic ions from the cytoplasmic compartment. The second component is associated with the fact that NaCl is an impermeable solute and therefore exerts an osmotic effect which draws water out of the cytoplasm; for this reason, the second component is also referred to as osmotic stress or turgor stress. Equivalent osmolar concentrations of various ionic and nonionic impermeable solutes, such as NaCl, K_2SO_4 and sucrose, will impose the same degree of this second component of water stress. The set of active processes carried out by organisms to cope with osmotic stress is defined as osmoregulation, central to which is the

* For correspondence. E-mail: shankar@ccmb.ap.nic.in.

Keywords. *Escherichia coli*; water activity; osmoregulation; anhydrotic stress; L-ornithine; uracil sensitivity.

restoration of osmotic balance and intracellular volume through the accumulation of nontoxic organic compatible solutes (Yancey *et al.* 1982; Le Rudulier *et al.* 1984; Csonka 1989; Csonka and Epstein 1996).

Although the component of salinity stress is well demarcated in plants (Greenway and Munns 1980; Wyn Jones 1984), yeast (Gaxiola *et al.* 1992; Posas *et al.* 1995), and bacteria such as *Bacillus subtilis* (Kunst and Rapoport 1995), *Staphylococcus aureus* (Bae *et al.* 1993), *Pseudomonas aeruginosa* (Shortridge *et al.* 1992) and the cyanobacteria (Hershkovitz *et al.* 1991; Fernandes *et al.* 1993), in *Escherichia coli* this component (as opposed to that of osmotic stress) does not appear to be prominent (Csonka and Epstein 1996). In other words, NaCl inhibits growth of *E. coli* only to the same extent as an equiosmolar concentration of an impermeable nonionic solute such as sucrose. Likewise, several adaptive phenomena associated with growth of *E. coli* in NaCl-containing media, including (i) the cytoplasmic accumulation of K^+ ions, L-glutamate, trehalose, glycine betaine and L-proline, (ii) the growth-promoting effects of externally supplied glycine betaine, dimethylthetin, ectoine, L-proline or choline, and (iii) transcriptional regulation of *ompF, ompC, proU, proP, bet, otsBA* and several *osm* loci, are elicited to an equivalent extent by nonionic impermeable solutes as well (reviewed in Csonka 1989; Csonka and Epstein 1996). The only phenomena so far known in *E. coli* that are elicited by NaCl but not by nonionic solutes during steady-state growth are the transcriptional induction of the *nhaA* (Karpel *et al.* 1991) and *kdp* (Gowrishankar 1985) operons.

An important prior condition in establishing that a particular physiological phenomenon is associated with osmotic stress (or with osmoregulation) is that the phenomenon should not be observed when one uses a freely permeable substance, such as glycerol, as the dissolved solute. Such an explicit distinction between the effect of impermeable solutes and that of permeable solutes has been described, for example, for sorbitol accumulation in cells of the mammalian renal medulla (Uchida *et al.* 1989), L-proline uptake in *S. aureus* (Bae and Miller 1992), osmoprotection by L-proline (Le Rudulier *et al.* 1982) and glycine betaine (Le Rudulier and Bouillard 1983) in *Klebsiella pneumoniae*, induction of pectate lyase in *Erwinia chrysanthemi* (Gouesbet *et al.* 1995), and the following phenomena in *E. coli*: cytoplasmic K^+ accumulation (Epstein and Schultz 1965), expression of energy-linked membrane functions (Houssin *et al.* 1991), and transcriptional control of the *kdp* (instantaneous induction; Laimins *et al.* 1981), *proU* (Gowrishankar 1985) and many *osm* (Gutierrez *et al.* 1987) genes.

In this paper we describe for the first time a set of physiological phenomena that are elicited to approximately the same extent by each of three solutes, NaCl (ionic, impermeable), sucrose (nonionic, impermeable) and glycerol (nonionic, permeable), added to the growth medium. As a consequence, these phenomena can be classified as neither chemical-specific nor osmotic-stress-related. We suggest that the common feature associated with use of the three solutes is that they lead to reduction in water activity of the cytoplasm (which is unaltered by the accumulation of compatible solutes in response to NaCl or sucrose addition), and have coined the term anhydrotic stress to refer to this distinct component of physiological water stress.

The phenomena that we have identified to be so associated in *E. coli* are intimately connected with the metabolism of L-arginine and the polyamines in this organism (reviewed in Glansdorff 1996). The shared biosynthetic pathway for these compounds is depicted schematically in figure 1. The *argA*-encoded acetylglutamate synthase reaction represents the first committed step in the pathway, and L-ornithine represents the branch-point intermediate which can be channelled either into polyamines via the activity of the enzyme ornithine decarboxylase or into L-arginine via its reaction with carbamoyl phosphate to form L-citrulline. Interestingly, carbamoyl phosphate is also required for biosynthesis of the pyrimidines, and synthesis of carbamoyl phosphate therefore is regulated independently and additively by the cytoplasmic concentrations of the respective end products, L–arginine and the pyrimidines. The *arg* biosynthetic genes constitute a regulon under repression control (in the presence of L-arginine) of the *argR* gene, and acetylglutamate synthase is subject also to feedback inhibition by L-arginine (Glansdorff 1996).

We found in this study that growth of wild-type *E. coli* is inhibited by uracil in media whose water activity had been

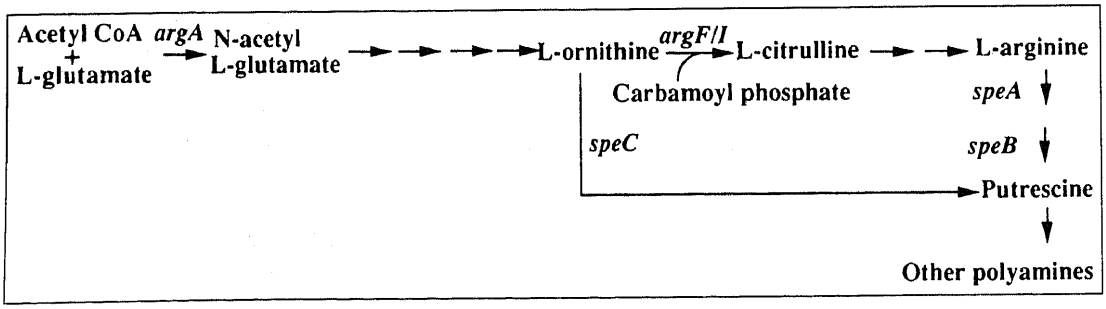

Figure 1. Pathway for biosynthesis of L-arginine and polyamines in *E. coli* (Glansdorff 1996). Each arrow represents one step in the pathway; only those genes and intermediates that are discussed in the text are identified.

Table 1. *E. coli* K-12 strains used.

Strain	Genotype*	Source or reference
CAG18475	*metC162*::Tn*10*	Singer *et al.* 1989
CAG18559	*nupG3157*::Tn*10*Kan	Singer *et al.* 1989
CSH141	Δ(*gpt-lac*)*5 supE rpsE*/[F′*lac*$^+$*pro*$^+$]	Miller 1992
MA5	*gal argA*	Celis 1977
MA255	*thr-1 leuB6 can-1 speB2 speC3 thi-1 relA17 lacY1 gal-6 xyl-7 mtl-2 rpsL133 tonA2 supE44*	Cunningham-Rundles and Maas 1975
MC4100	Δ(*argF-lac*)*U169 rpsL150 araD139 relA1 flbB5301 deoC1 ptsF25 rbsR*	Gowrishankar 1985
MG1655	wild type	Singer *et al.* 1989
PL8-31	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 supE44 galK2 hisC3 rfbD1 metG87 serA25 metK86* Δ(*speC-glc*)*63 rpsL25 kdgK51 xylA5 mtl-1 thi-1*	Hunter *et al.* 1975
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)*461*	Gowrishankar 1985
GJ1201	GJ134 *argA202*	This study
GJ1201K	GJ134 *argA202 zga-900*::Tn*10*dKan	This study
GJ1201T	GJ134 *argA202 zga-901*::Tn*10*dTet	This study
GJ1205	GJ134 *recD1901*::Tn*10*	This study
GJ1206	GJ134 *argA202 recD1901*::Tn*10*	This study
GJ1217	MC4100 *argA202 recD1901*::Tn*10*	This study
GJ1222	MG1655 *argA202 recD1901*::Tn*10*	This study
GJ1263	*thi-1 argR64 zha-901*::Tn*10*dKan *recD1901*::Tn*10*	This study
GJ1264	*thi-1 argR64 argA202 zha-901*::Tn*10*dKan *recD1901*::Tn*10*	This study
GJ1266	MC4100 *argR64 zha-901*::Tn*10*dKan	This study
GJ1268	MC4100 *argA202 argR64 recD1901*::Tn*10 zha-901*::Tn*10*dKan	This study
GJ1273	GJ134 *argA recD1901*::Tn*10*	This study
GJ1274	GJ134 *argA recD1901*::Tn*10* AcGlt$^+$	This study
GJ1275	GJ134 AcGlt$^+$	This study
GJ1276	MA255 *metC162*::Tn*10*	This study
GJ1293	MC4100 *speC3*	This study
GJ1294	PL8–31 *metC162*::Tn*10*	This study
GJ1296	MC4100 Δ(*speC-glc*)*63*	This study

*Gene designations are as described in Berlyn *et al.* 1996. All strains are F$^-$ unless otherwise indicated. The *argR64, recD1901*::Tn*10* and *zha-901*::Tn*10*dKan mutations were sourced from strains MA1030 (Coli Genetic Stock Center), CAG12135 (Singer *et al.* 1989) and GJ912 (Saroja and Gowrishankar 1996) respectively. The Δ*speC* mutation in PL8-31 was earlier called *glc-1* (Hafner *et al.* 1977).

reduced by either impermeable or permeable dissolved solutes, and that a newly isolated *argA* bradytrophic mutation relieved this inhibition. Mutations in *argR*, or supplementation of the medium with L-arginine or L-citrulline, also conferred relief from uracil sensitivity in the low-water-activity media. On the other hand, mutations in *speC*, or supplementation with L-ornithine, led to an exacerbation of growth inhibition under these conditions. We interpret these results in terms of a model in which *E. coli* growth under conditions of anhydrotic stress is inhibited by a high endogenous pool of L-ornithine.

Materials and methods

Bacterial strains, plasmids and phages: *E. coli* K-12 strains used in this study are listed in table 1. Plasmids employed included the vector pBR322 (Sambrook *et al.* 1989), and its derivative pODC carrying the cloned *speC*$^+$ gene (Boyle *et al.* 1984). Phage P1 was from our laboratory stock. The phages λ1098 and λ1105, used to generate random transpositions of Tn*10*dTet and Tn*10*dKan respectively, have been described (Miller 1992).

Growth media and conditions: Defined and nutrient media were, respectively, minimal A (supplemented with 0.2% glucose or other indicated carbon source, and the appropriate auxotrophic requirements) and Luria–Bertani (LB) medium (Miller 1992). Uracil, tetracycline (Tet), kanamycin (Kan) and streptomycin were used at final concentrations of 40, 15, 40 and 100 μg ml^{-1}, respectively. Unless otherwise indicated, the growth temperature was 37°C. For growth rate measurements, cultures were started with an initial inoculum of 1 : 1000 or less, followed by uninterrupted incubation in a rotary water bath shaker set at 200 rpm; at the end of the experiment, cultures were

checked by streaking on plates to ensure that faster-growing mutants had not been selected under the conditions.

Transposon tagging of argA202 mutation in GJ1201: Random transpositions of Tn*10*dKan or Tn*10*dTet into the chromosome of the NaCl-tolerant mutant GJ1201 were generated following infection of the strain with vectors λ1105 or λ1098 respectively, as described (Miller 1992). Phage P1 lysates prepared on the pools of Kan^r and Tet^r clones were used to infect the wild-type parent GJ134, and a double selection was imposed by plating for transductants on 0.8 M NaCl-containing minimal A plates supplemented with Kan or Tet (as appropriate) and glycine betaine. P1 lysates prepared on individual colonies purified from these plates were then used to determine linkage between each Kan^r or Tet^r insertion and the mutation conferring NaCl tolerance in GJ1201. In this manner, one Kan^r insertion and one Tet^r insertion (in strains GJ1201K and GJ1201T respectively) were identified that were 70% cotransducible with each other and that were, respectively, 10% and 6% linked to the *argA202* mutation in GJ1201. Based on the mapping data below, the two insertion alleles have been designated as *zga-900*:: Tn*10*dKan and *zga-901*:: Tn*10*dTet respectively.

Construction of isogenic argA auxotrophs GJ1274 and GJ1275: Strain GJ1273 is an *argA* auxotrophic derivative of GJ134 (with the *argA* mutation obtained from MA5), but is unable to utilize exogenous *N*-acetylglutamate for L-arginine synthesis presumably because of an inability to transport the compound. We selected for spontaneous mutants ($AcGlt^+$, genetic locus uncharacterized) of GJ1273 that were now able to use *N*-acetylglutamate for satisfying the auxotrophic requirement, and designated one of them GJ1274. An $argA^+$ transductant of GJ1274 was designated GJ1275.

Construction of isogenic speC and speC$^+$ strains: In preliminary transduction experiments into the *speB speC* (putrescine-auxotrophic) strain MA255, we established that the *metC162*:: Tn*10* (in CAG18475) and *nupG3157*:: Tn*10*Kan (in CAG18559) insertions are, respectively, 4% and > 99% linked to *speC*. A *nupG3157*:: Tn*10*Kan derivative of MC4100 was used as recipient in two tranductions to Tet^r; the donors were GJ1276 and GJ1294, which had *metC*:: Tn*10* linked to *speC3* and Δ*speC* respectively. The Tet^r colonies from each cross were scored for Kan^s, and the latter were inferred to have inherited the cognate donor *speC* mutant allele (given the extremely close linkage between *nupG* and *speC*). The *metC*:: Tn*10* marker was then crossed out from the *speC* mutant strains by transduction to Met^+. The resultant strains GJ1293 (*speC3*) and GJ1296 (Δ*speC*) are thus isogenic derivatives of MC4100 ($speC^+$).

Other genetic techniques: The methods for transformation (Sambrook *et al.* 1989), nitrosoguanidine mutagenesis (Miller 1992), and transduction with phage P1 (Gowrishankar 1985) have been described. F′ *lac pro* exconjugants of MC4100, GJ1217 (*argA202*) and GJ1266 (*argR64*) were obtained following conjugation with CSH141 as donor, with selection for Lac^+ streptomycin-resistant colonies (Miller 1992); these exconjugants were used for growth experiments involving choline supplementation of low-water-activity media [because choline uptake and oxidation to glycine betaine is governed by the *bet* genes which are located in the *argF–lac* interval deleted in MC4100 derivatives and carried on F′ *lac pro* (Le Rudulier *et al.* 1984)]. Transduction of *argA* and *argR* alleles was achieved with the aid of the linked transposon markers *recD1910*:: Tn*10* and *zha-901*:: Tn*10*Kan respectively. Control experiments indicated that the transposon markers had no effect on growth phenotypes in low-water-activity media (data not shown).

N-Acetylglutamate synthase assays: Crude cell extracts for enzyme assay were prepared from cultures grown in glucose–minimal A–uracil as described (Gowrishankar and Pittard 1982), with the modification that harvested cells, after washing, were resuspended in 5 ml of acetylglutamate synthase extraction buffer (Leisinger and Haas 1975) prior to passage through a French pressure cell.

The method for assay of *N*-acetylglutamate synthase was based on that described earlier (Haas *et al.* 1972). The reaction mixture contained, in a total volume of 50 μl: Tris–HCl (pH 9), 200 mM; $MgCl_2$, 10 mM; acetyl CoA, 4.6 mM; L-[U-^{14}C]-glutamic acid (Bhabha Atomic Research Centre, Mumbai) [pH adjusted to 9 with KOH and specific activity adjusted to 0.4 μCi (1.5×10^4 Bq) μmol^{-1}], 10 mM; and cell extract, 25 μl. After incubation at room temperature for 10 min, the reaction was terminated by the addition of 0.1 ml of 0.3 N HCl. The entire mixture was then loaded on a 1.5-ml cation exchange resin column (Dowex 50W-X8, 50–100 mesh, H^+ form, equilibrated with 0.1 M HCl) and eluted with four successive washes each with 1 ml of 0.1 M HCl. Each of the four eluate fractions was collected in 15 ml of scintillation fluid (Bray 1990), and the radioactivity measured in a Hewlett-Packard scintillation counter. The sum of the radioactivity in the four fractions, after correction for the blank value, in which the sample had been processed in the same manner except that acetyl CoA was omitted from the reaction mixture, was taken as a measure of the amount of *N*-acetylglutamate formed in the reaction.

Protein concentrations in the cell extracts were determined by the method of Bradford (1976), and enzyme specific activity values are reported as cpm of radioactivity incorporated into the product per minute per mg protein in the crude extracts.

Results

Isolation of NaCl-tolerant mutant GJ1201 and uracil dependence of growth phenotype

The starting point for this study was the isolation, from a nitrosoguanidine-mutagenized cell population of *E. coli*

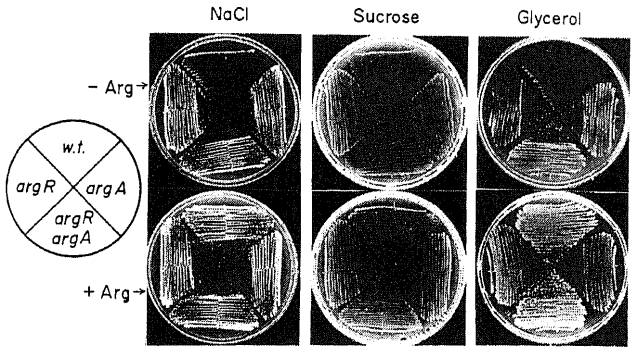

Figure 2. Growth of wild-type, *argA202* (*argA*) and *argR64* (*argR*) strains on agar plates of low water activity. Isogenic strains were streaked as depicted in the template on the left: MC4100 (w.t.), GJ1217 (*argA*), GJ1266 (*argR*), and GJ1268 (*argA argR*). Glucose–minimal A–uracil agar plates supplemented, as marked, with NaCl (at 0.5 M), sucrose (at 0.75 M), or glycerol (at 1.65 M), and additionally without (top row) or with (bottom row) 1 mM L-arginine, were used. The pairs of plates (for the three solutes) were incubated for 42 to 60 hours prior to photography. Note cross-feeding for growth of the wild-type strain by the *argR* but not the *argA* mutant in the top row of plates.

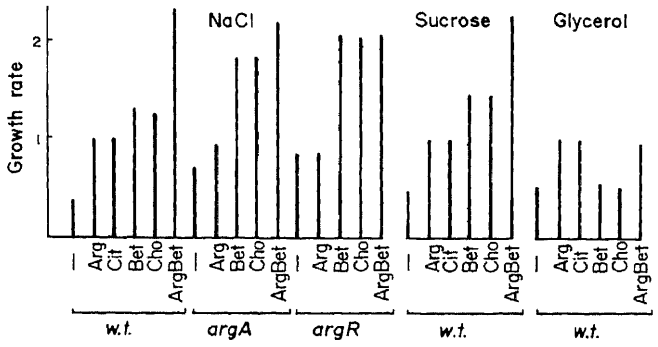

Figure 3. Growth rates of wild-type (w.t.; MC4100), *argA202* (*argA*; GJ1217) and *argR64* (*argR*; GJ1266) strains in glucose–minimal A–uracil liquid cultures containing NaCl (0.5 M), sucrose (0.9 M) or glycerol (1.4 M) and either unsupplemented (–) or additionally supplemented with one of the following (each at 1 mM): L-arginine (Arg), L-citrulline (Cit), glycine betaine (Bet), choline (Cho), or L-arginine + glycine betaine (ArgBet). Strain derivatives carrying F′ *lac pro* were used for the experiments with choline supplementation (Le Rudulier *et al.* 1984). Growth rates shown in the histogram have been normalized against the value (taken as 1.0) for one of the three L-arginine-supplemented cultures of the wild-type strain, in medium containing NaCl, sucrose or glycerol as appropriate. Actual growth rates (generations per hour) for these three reference cultures were 0.26, 0.16 and 0.26 respectively.

strain GJ134, of a mutant GJ1201 which exhibited enhanced NaCl tolerance in glucose–minimal A–uracil medium (data not shown, but see figures 2 and 3). New Tet^r and Kan^r insertions linked to the mutation in GJ1201 were obtained as described above, and were used with the panel of mapping strains of Singer *et al.* (1989) to localize the mutation to 60 min on the *E. coli* linkage map, 99% cotransducible with a *recD*::Tn*10* insertion (data not shown).

A derivative of MC4100 (designated GJ1217) carrying the mutation from GJ1201 was constructed by transduction with the linked *recD*::Tn*10* marker. Strain MC4100 is an ancestor of GJ134 (the latter having additional mutations in *putPA*, *proP* and *pyr* loci). It was observed that GJ1217 was more NaCl-tolerant than MC4100 only in uracil-supplemented medium (figures 2 and 3), and not in medium unsupplemented with uracil (data not shown). We were able to attribute this difference to the fact that the growth of MC4100 was substantially retarded in the former medium compared to that in the latter. For example, the highest NaCl concentration that permitted growth of strain MC4100 on glycine-betaine-supplemented glucose–minimal A plates (in three days) was reduced from 1.1 M in the absence of uracil to 0.7 M in its presence, whereas it remained at 1.1 M for GJ1217 in both media. (As described below, glycine betaine addition by itself does not influence either the uracil-sensitivity phenotype or its alleviation by the GJ1201 mutation.) Virtually identical phenotypic correlations were reproduced in another *E. coli* 'wild-type' strain, MG1655, and its derivative GJ1222 into which the mutation from GJ1201 had been transduced (data not shown).

Consistent with earlier results (Piérard *et al.* 1965; Jensen 1993), uracil addition to glucose–minimal medium not supplemented with NaCl did not inhibit growth of the wild-type strains MC4100 or MG1655 (data not shown). Thus it is the combination of NaCl and uracil that appears to be growth-inhibitory to wild-type *E. coli* and the mutation in GJ1201 alleviates this effect. For convenience, this phenotype is referred to as uracil–NaCl tolerance/sensitivity in some of the sections below. The identification of GJ1201 as an NaCl-tolerant mutant may therefore be considered fortuitous in that the parent GJ134 is a pyrimidine auxotroph so that the mutant selections had been done in uracil-containing medium.

In light of the knowledge that osmoprotectants such as glycine betaine and choline alleviate NaCl-imposed growth inhibition in *E. coli* (Le Rudulier *et al.* 1984; Csonka 1989; Csonka and Epstein 1996), we examined the effect of these compounds on uracil–NaCl tolerance (figure 3). (The choline supplementation experiments were done with derivatives of MC4100 and GJ1217 each carrying F′ *lac pro*, so as to make them *bet*$^+$.) As expected, each of the two compounds by itself increased the growth rate of the wild-type MC4100 derivative in NaCl-supplemented medium; nevertheless, the growth advantage of GJ1217 over MC4100 continued to be maintained on these media (figure 3). These data indicate that the osmoprotective effect of the two compounds is additive to, and independent of, the mechanism conferring uracil–NaCl tolerance in GJ1217.

Identification of GJ1201 as argA bradytroph

In view of the close linkage of the mutation in GJ1201 with *recD*, we tested whether *argA* (which is adjacent to *recD*) may be the candidate mutant locus. The following experiments led us to conclude that the mutation conferring

Table 2. *N*-Acetylglutamate synthase activity in *argA202* mutants.

Strain	Relevant genotype	Specific activity*
GJ1205	*argA*$^+$ *argR*$^+$	1958
GJ1206	*argA202 argR*$^+$	151
GJ1263	*argA*$^+$ *argR64*	9403
GJ1264	*argA202 argR64*	343

*Enzyme specific activities were measured in cultures grown to mid-exponential phase in glucose–minimal A–uracil medium, and are expressed as cpm of radioactivity incorporated into *N*-acetylglutamate per min per mg protein.

uracil–NaCl tolerance in GJ1201 is a bradytrophic mutation in *argA*, which we have designated *argA202*.

(i) In comparison with its isogenic *argA*$^+$ derivative GJ1205, the *argA202* mutant GJ1206 was hypersensitive to the L-arginine analogue L-canavanine (at 10 μg ml^{-1}) at 37°C, and conditionally auxotrophic for L-arginine at 42°C. Interestingly, the latter requirement was suppressed by uracil–NaCl, and GJ1206 exhibited enhanced uracil–NaCl tolerance even at 42°C (data not shown).

(ii) Growth of the *argA202* mutant was also inhibited by the addition of 40 μg ml^{-1} each of L-cysteine and L-methionine to minimal medium, and this inhibition was relieved upon supplementation with L-arginine. We were able to demonstrate that this phenotype (Cys–Met inhibition) was also dependent on the *relA* mutation in GJ1206, and is not exhibited in *relA*$^+$ derivatives of the strain. Although the molecular mechanism underlying Cys–Met inhibition is not known, we speculate that it reflects an inhibition of L-arginine synthesis in the *argA202* mutant, which is alleviated by ppGpp-mediated stimulation of *argA* transcription in the *relA*$^+$ derivatives (Kelker and Eckhardt 1977). RelA-dependent growth perturbations by L-cysteine have been reported earlier (Harris 1981; Sorensen and Pedersen 1991). We were also able to exploit the phenotype of Cys–Met inhibition in GJ1206 to isolate revertants and thus to demonstrate that a single mutation tightly linked to *recD*::Tn*10* is responsible for the phenotype of uracil–NaCl tolerance, L-canavanine hypersensitivity, conditional arginine auxotrophy and Cys–Met inhibition in this strain (data not shown).

(iii) Acetylglutamate synthase activity in the *argA202* mutant was considerably less than that in the wild-type strain. The difference in activity between *argA*$^+$ and *argA202* was more pronounced in an *argR* background than in *argR*$^+$ strains (table 2), presumably because of the physiological derepression of the biosynthetic pathway in the bradytrophic *argR*$^+$ mutant. The extent of inhibition of enzyme activity caused by addition of 1 mM L-arginine was the same in cell extracts of both pairs of strains (data not shown).

(iv) Finally, a strain (GJ1274) carrying a known *argA* auxotrophic mutation and constructed as described in Materials and methods, also displayed enhanced uracil–NaCl tolerance in comparison with its isogenic *argA*$^+$ derivative GJ1275, when tested on glucose–minimal A plates supplemented with *N*-acetylglutamate (data not shown).

Other links between L-arginine metabolism and uracil–NaCl tolerance

We next explored the relation between uracil–NaCl tolerance and other perturbations in L-arginine metabolism. Introduction of the *argR64* mutation, or supplementation of the medium with 1 mM L-arginine, restored uracil–NaCl tolerance to MC4100 to much the same extent as the *argA202* mutation (figures 2 and 3). The growth-promoting effect of glycine betaine on the wild-type strain was additive to that of both the *argR*64 mutation and L-arginine supplementation; likewise, growth promotion by choline was additive to that of *argR64* (tested in the *bet*$^+$ F′ *lac pro* derivative) (figure 3). The *argR64* (but not *argA202*) derivative was able to cross-feed MC4100 for restoration of uracil–NaCl tolerance (figure 2), presumably through excretion of L-arginine. That the three perturbations are acting through a common mechanism was indicated by the data that combinations of them (*argR64 argA202* double mutant, or the mutants grown on L-arginine-supplemented medium) were not more growth-enhancing on the NaCl-containing medium than any one taken alone (figures 2 and 3). A second *argR* mutation (*argR203*) newly obtained in this study also behaved exactly like *argR64* in these experiments (data not shown). Neither *argR* nor L-arginine supplementation had any significant effect on growth of MC4100 in minimal medium without NaCl supplementation (with or without uracil). Like L-arginine, exogenous L-citrulline also increased the growth rate of MC4100 on NaCl-supplemented glucose–uracil–minimal medium (figure 3).

On the other hand, addition of 1 mM L-ornithine inhibited growth of MC4100 on uracil–minimal A medium even with moderate (0.4 M) NaCl supplementation (figure 4, compare growth between second and third panels of top row; measured doubling times of 110 and >240 min respectively without and with 1 mM L-ornithine). That is, L-ornithine addition apparently serves to accentuate uracil–NaCl sensitivity in the wild-type strain. Once again, relief from such sensitivity was observed with exogenous L-arginine supplementation, or upon introduction of the *argR* or *argA202* mutations (data not shown).

An incubation temperature of 37°C or higher was necessary for demonstration of all the growth phenotypes described. We also observed that uracil–NaCl tolerance associated with *argR*, *argA202* or exogeneous L-arginine was prominent only when the osmolar concentration of the medium exceeded a certain threshold (corresponding to >0.5 M added NaCl) (data not shown). Indeed, because of

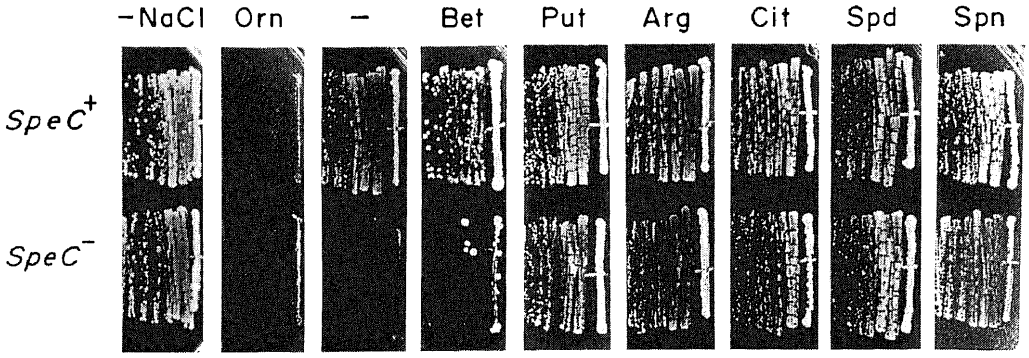

Figure 4. Growth of MC4100 (*speC*$^+$) and GJ1293 (*speC*) strains on glucose–minimal A–uracil agar plates supplemented (except extreme left) with 0.4 M NaCl and either no other substance (–) or one of the following (at 1 mM; as marked on the top of each panel): L-ornithine (Orn), glycine betaine (Bet), putrescine (Put), L-arginine (Arg), L-citrulline (Cit), spermidine (Spd), or spermine (Spn). Plates were incubated for 28 hours before photography. The panel on the extreme left shows growth of the two strains on minimal A plates not supplemented with NaCl.

this threshold effect, the phenotype of enhanced uracil–NaCl tolerance associated with these perturbations is more readily demonstrable in glycine-betaine-supplemented media wherein the baseline tolerance of the wild-type strain itself is around 0.65 M NaCl.

Effects of speC gene dosage on uracil–NaCl sensitivity

As discussed below, the findings so far described led us to consider a tentative model in which sensitivity or tolerance to uracil–NaCl was associated, respectively, with an increase or decrease in size of the endogenous L-ornithine pool. The *speC* gene encodes the enzyme ornithine decarboxylase which catalyses the conversion of L-ornithine to putrescine (figure 1), and we therefore examined the effects of modifications in *speC* gene dosage on the uracil–NaCl sensitivity and tolerance phenotypes.

For this purpose, we first compared the growth behaviour of a *speC3* mutant strain (GJ1293) with that of its isogenic *speC*$^+$ parent MC4100. Both strains grew equally well on NaCl-unsupplemented glucose–minimal A–uracil medium (compare top and bottom rows of the extreme left panel in figure 4), and also on uracil-unsupplemented glucose–minima A–NaCl media (data not shown). Growth of the *speC* mutant was, however, significantly retarded in medium supplemented with both uracil and a moderate concentration (0.4 M) of NaCl (figure 4). Addition of glycine betaine served to enhance the difference in growth between the *speC*$^+$ and *speC* strains, ostensibly by promoting growth of the former while leaving unaffected the growth retardation in the latter (figure 4; measured doubling times of 70 min and >480 min respectively for the two strains). The growth inhibition of the *speC3* mutant was relieved by supplementation with L-arginine or L-citrulline (but not L-ornithine) and also, interestingly, by supplementation with putrescine or the other polyamines spermidine and spermine (figure 4). The growth inhibition of the *speC3* strain was also similarly relieved by introduction of the *argR* or *argA202* mutations (data not shown). To exclude the possibility that the observed phenomena are idiosyncratic for the *speC3* allele, we repeated the experiments with an isogenic Δ*speC* strain (GJ1296), and obtained identical results (data not shown).

In the converse set of experiments, we examined the effect of multicopy *speC*$^+$ on the uracil–NaCl sensitivity phenotype. Strain MC4100 was transformed with either plasmid pODC (carrying the *speC*$^+$ gene) or the vector pBR322 (as control). The data from the growth rate comparisons allowed us to conclude that plasmid pODC was just as effective as L-arginine supplementation or the *argA202* or *argR* mutations in conferring uracil–NaCl tolerance to MC4100 (data not shown).

Equivalent effects upon substitution of NaCl by sucrose or glycerol

We observed that correlations identical to those described above, between perturbations of the biosynthetic pathway for L-arginine and polyamines on the one hand and growth behaviour of strains on media supplemented with uracil and NaCl on the other, could be established also when NaCl was substituted by sucrose or even glycerol in the culture media (figures 2 and 3). Thus, whereas the wild-type strain MC4100 was inhibited by equiosmolar concentrations of NaCl or sucrose and by glycerol at a higher concentration in glucose–uracil–minimal medium, introduction of the *argA202* or *argR* mutations or supplementation with L-arginine or L-citrulline conferred protective effects in all the three media. Cross-feeding for growth of the wild-type strain by the *argR* mutant was also observed on the sucrose-supplemented and glycerol-supplemented plates (figure 2). Addition of K_2SO_4 (0.35 M), or of mixtures of solutes (such as 0.4 M NaCl or 0.5 M sucrose with 1 M glycerol, or 0.3 M NaCl with 0.6 M sucrose), elicited the same growth patterns with the different strains as those depicted in figure 2 (data not shown). In a control experiment, we found no induction of the osmo-responsive *proU* operon even at the highest concentration of

glycerol tested that permitted growth (data not shown), confirming that glycerol did not exert any osmotic effect on the cells under these conditions (Gowrishankar 1985).

Data from growth-rate experiments in the (uracil-supplemented) liquid cultures (figure 3) also indicated that: (i) osmoprotective substances such as glycine betaine or choline (the latter tested with a F′ *lac pro* derivative) promote growth of the wild-type strain only on the NaCl-supplemented or sucrose-supplemented medium, whereas L-arginine or L-citrulline do so on all three media; and (ii) the growth-promoting effect of glycine betaine is additive to that of L-arginine, *argA202* or *argR* in both the NaCl-supplemented and the sucrose-supplemented cultures.

In the same context and by experiments similar to those illustrated in figure 4, we could also demonstrate that the growth retardation associated with both L-ornithine addition and the *speC* mutations were reproduced in glucose – minimal A – uracil media supplemented with 0.7 M sucrose or 0.7 M glycerol instead of 0.4 M NaCl (data not shown).

Discussion

The salient findings of this study included the following: (i) inhibition by uracil of growth of wild-type *E. coli* strains in media supplemented with NaCl, sucrose or glycerol; (ii) relief from such inhibition by any one of the following: introduction of mutations in *argA* or *argR*, exogenous supplementation with L-arginine or L-citrulline, or introduction of a multicopy *speC*$^+$ plasmid; and (iii) accentuation of growth inhibition by exogenous L-ornithine or by loss-of-function mutations in *speC*. In the sections below, we first discuss the implications of the fact that the observed phenomena are elicited in the presence of either impermeable or permeable dissolved solutes. We then go on to propose a correlation between the conditions associated with relief of uracil sensitivity in low-water-activity media on the one hand and a decrease in the levels of cytoplasmic L-ornithine on the other.

Identification of anhydrotic stress as a new component of biological water stress

As described in Introduction, earlier studies have identified salinity (or ionic) stress and osmotic (or turgor) stress as the components that contribute to growth inhibition in media of low water activity. By these criteria, dissolved solutes that are nonionic and freely permeable (such as glycerol) are expected not to exert any physiological water stress.

The finding in this study that some NaCl-elicited phenomena in *E. coli* are also elicited by sucrose and by glycerol therefore represents the first identification of a novel component of stress associated with growth at low water activity. That each of the three solutes, above a particular concentration, inhibits growth of wild-type *E. coli* is by itself not an indication that they are doing so through a common mechanism; rather it is the fact that we could identify single mutations such as *argA202* or *argR* which relieve the growth inhibition by all three solutes, and others such as *speC* which accentuate it, that allows us to infer the existence of such a common mechanism.

We suggest that the absolute value of cytoplasmic water activity also influences cell physiology, and propose the term anhydrotic stress for the purpose. Several studies have earlier described the effects of the chemical water potential on functions of various proteins *in vitro* (reviewed in Leikin *et al.* 1993; Parsegian *et al.* 1995), but similar *in vivo* effects had not been explicitly postulated. The process or set of processes by which organisms adapt to this novel component of water stress may be called anhydrotolerance.

Intracellular accumulation of compatible solutes, in response to the presence of impermeable dissolved solutes in the growth medium, is expected to contribute to osmoregulation (by restoring osmotic balance) but not to confer anhydrotolerance. Likewise, permeable dissolved solutes will contribute to anhydrotic stress but not osmotic stress. These features may provide an explanation for our observations that a higher osmolar concentration of glycerol than of NaCl or sucrose is required for exhibition of the growth-sensitivity phenotype (figure 3), since only the latter two compounds are expected to impose turgor stress in addition to anhdyrotic stress. Furthermore, it is known that supplementation with impermeable solutes (but not permeable solutes) is associated with cytoplasmic K^+ accumulation (Epstein and Schultz 1965), and K^+ ions have been shown to inhibit ornithine decarboxylase activity (Rubenstein *et al.* 1972); this effect may also contribute to an exacerbation of anhydrotic stress with NaCl or sucrose (see below). Indeed, in media supplemented with glycine betaine (in which there is known to be substantial physiological alleviation of turgor stress, and decrease in cytoplasmic [K^+]; Sutherland *et al.* 1986), equivalent osmolar concentrations of each of the three solutes inhibited growth of the wild-type strain on uracil-supplemented medium to approximately the same extent (data not shown; see figure 3).

Evidence for correlation between growth inhibition and high cytoplasmic L-ornithine pool

When considered together, the various conditions that alleviate uracil sensitivity in low-water-activity medium are associated, seemingly paradoxically, with both increase (*argR*, L-arginine/L-citrulline addition) and decrease (*argA*) in the cellular L-arginine pool, and also with both increase (*argR*) and decrease (*argA*, L-arginine/L-citrulline addition) in the metabolite flux through the arginine biosynthetic pathway. In our search for a possible mechanism, we were therefore led to consider the one apparently common correlate to all of the alleviating conditions above: a decreased cytoplasmic pool size of L-ornithine.

Earlier studies have established such a correlation both for L-arginine supplementation (through ArgR-mediated repression of the pathway; Morris and Koffron 1969; Glansdorff

1996), and for *argR* mutations (through competition for L-ornithine from the enormously derepressed ornithine carbamoyltransferase isoenzymes encoded by the *argF* and *argI* genes; Morris and Koffron 1969). The bradytrophic *argA202* mutation is also expected to reduce metabolite flux through the arginine biosynthetic pathway.

By itself uracil addition is known to repress carbamoyl phosphate synthesis and consequently to derepress the arginine biosynthetic enzymes (because of reduction in flux at the step of ornithine transcarbamoylation, and a consequent reduction in size of the L-arginine pool) (Gorini and Kalman 1963; Piérard *et al.* 1965; Glansdorff 1996). Thus the intracellular L-ornithine pool in the wild-type strain is expected to be larger in uracil-supplemented medium, which may account for our observation that uracil is necessary in the growth medium for exhibition of the various phenomena described above. Consistent with these suggestions, we have also observed that MC4100 is more sensitive to L-canavanine in glucose–minimal A medium supplemented with uracil than in medium not so supplemented (data not shown). Nevertheless, anhydrotolerance associated with L-arginine addition appears to be an absolute effect (and not merely the relief of uracil-mediated inhibition), since the maximum tolerated NaCl concentration for growth of wild-type strains such as MC4100 or MG1655 even on uracil-free glucose–minimal A agar plates (in the presence of glycine betaine) was increased from around 0.9 M in the absence of L-arginine to around 1.1 M in its presence (data not shown).

Our finding that exogenously provided L-ornithine and L-citrulline (which are adjacent intermediates in the arginine biosynthetic pathway) have diametrically opposite effects on uracil sensitivity of the wild-type strain in low-water-activity media provides support to the correlation proposed above. Likewise, the observations that the uracil-sensitivity phenotype is accentuated or attenuated, respectively, by *speC* mutations or multicopy *speC*$^+$ may be understood in light of the role expected to be played by the enzyme ornithine decarboxylase in modulating the endogenous pool size of L-ornithine. The effect of the polyamines in relieving growth inhibition of the *speC* mutant (figure 4) may also be explained by the fact that the addition of exogenous polyamines leads to a reduction in endogenous L-ornithine levels (Cataldi and Algranati 1989).

In support of the possibility that the correlation postulated above between decreased L-ornithine levels and anhydrotolerance is valid in other systems, we have observed that exogenous L-arginine confers anhydrotolerance in the following Gram-negative and Gram-positive bacteria, inclusive of both those that use the linear and those that use the cyclic pathway of L-arginine biosynthesis (Cunin *et al.* 1986; NaCl concentrations used for testing mentioned in parentheses): *Salmonella typhimurium* (0.8 M), *Pseudomonas aeruginosa* (0.5 M), and *Bacillus subtilis* (1.4 M).

The exact mechanism by which L-ornithine and low water activity together mediate inhibition of growth is not clear. It is known that a build-up of the cytoplasmic L-ornithine level to a sufficiently high concentration is toxic to *E. coli* (Crabeel *et al.* 1975; Cataldi and Algranati 1989). However, unlike the situation observed with exogenous cyanate addition which has also been reported to induce a uracil-sensitive growth phenotype (Guilloton and Karst 1987), this L-ornithine-mediated toxicity is not likely to be the consequence of an inhibitory effect on carbamoyl phosphate synthesis, because it can be demonstrated even in mutants defective in the gene for carbamoyl phosphate synthase (Crabeel *et al.* 1975). Two alternative possibilities (not mutually exclusive) may therefore be considered for the growth inhibition observed in this study: (i) that, under conditions of anhydrotic stress, endogenous L-ornithine builds up to reach levels that are toxic; or (ii) that a given (unchanged) endogenous level of L-ornithine becomes more toxic when combined with the stress of a reduced water activity of the cytoplasm.

Finally, it is worth noting that several earlier studies have established an inverse correlation between intracellular polyamine need and content on the one hand and osmolarity of the growth medium on the other in both microorganisms (Mager 1955; Munro *et al.* 1972; Munro and Bell 1973; Munro and Sauerbier 1973; Capp *et al.* 1996) and mammalian cells (Munro *et al.* 1975; Perry and Oka 1980; Käpyaho and Jänne 1982; Poulin *et al.* 1991). The connection, if any, between our present findings and this earlier body of work needs to be further explored.

Acknowledgements

We thank B. J. Bachmann, M. K. B. Berlyn, R. T. F. Celis and C. A. Gross for strains, K. Igarashi for plasmid pODC, and D. Balasubramanian, L. N. Csonka and D. P. Kasbekar for discussions.

This work was supported by the Departments of Science and Technology and of Biotechnology, Government of India. J. G. is Honorary Senior Fellow of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India.

References

Bae J. -H. and Miller K. J. 1992 Identification of two proline transport systems in *Staphylococcus aureus* and their possible roles in osmoregulation. *Appl. Environ. Microbiol.* **58**, 471–475.

Bae J. -H., Anderson S. H. and Miller K. J. 1993 Identification of a high-affinity glycine betaine transport system in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **59**, 2734–2736.

Berlyn M. K. B., Low K. B., Rudd K. E. and Singer M. 1996 Linkage map of *Escherichia coli* K-12, edition 9. In *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd edn. (ed. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger), pp. 1715–1902. ASM Press, Washington, DC.

Boyle S. M., Markham G. D., Hafner E. W., Wright J. M., Tabor H. and Tabor C. W. 1984 Expression of the cloned genes encoding the putrescine biosynthetic enzymes and methionine adenosyltransferase of *Escherichia coli (speA, speB, speC and metK)*. *Gene* **30**, 129–136.

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.

Bray G. A. 1990 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**, 279–285.

Capp M. W., Cayley D. S., Zhang W., Guttman H. J., Melcher S. E., Saecker R. M., Anderson C. F. and Record M. T. Jr. 1996 Compensating effects of opposing changes in putrescine (2+) and K^+ concentrations on *lac* repressor–*lac* operator binding: *in vitro* thermodynamic analysis and *in vivo* relevance. *J. Mol. Biol.* **258**, 25–36.

Cataldi A. A. and Algranati I. D. 1989 Polyamines and regulation of ornithine biosynthesis in *Escherichia coli. J. Bacteriol.* **171**, 1998–2002.

Celis T. F. R. 1977 Properties of an *Escherichia coli* K-12 mutant defective in the transport of arginine and ornithine. *J. Bacteriol.* **130**, 1234–1243.

Crabeel M., Charlier D., Cunin R., Boyen A., Glansdorff N. and Piérard A. 1975 Accumulation of arginine precursors in *Escherichia coli:* effects on growth, enzyme repression, and application to the forward selection of arginine auxotrophs. *J. Bacteriol.* **123**, 898–904.

Csonka L. N. 1989 Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **53**, 121–147.

Csonka L. N. and Epstein W. 1996 Osmoregulation. In *Escherichia coli and Salmonella: cellular and molecular biology,* 2nd edn. (ed. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger), pp. 1210–1223. ASM Press, Washington, DC.

Cunin P., Glansdorff N., Piérard A. and Stalon V. 1986 Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* **50**, 314–352.

Cunningham-Rundles S. and Maas W. K. 1975 Isolation, characterization, and mapping of *Escherichia coli* mutants blocked in the synthesis of ornithine decarboxylase. *J. Bacteriol.* **124**, 791–799.

Epstein W. and Schultz S. G. 1965 Cation transport in *Escherichia coli.* V. Regulation of cation content. *J. Gen. Physiol.* **49**, 221–234.

Fernandes T., Iyer V. and Apte S. K. 1993 Differential effects of salt and osmotic stress on growth and nitrogen fixation in *Anabaena* sp. strain L-31. *Appl. Environ. Microbiol.* **59**, 899–904.

Gaxiola R., de Larrinoa I.F., Villalba J.M. and Serrano R. 1992 A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* **11**, 3157–3164.

Glansdorff N. 1996 Biosynthesis of arginine and polyamines. In *Escherichia coli and Salmonella: cellular and molecular biology,* 2nd edn. (ed. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umbarger), pp. 408–433. ASM Press, Washington, DC.

Gorini L. and Kalman S. M. 1963 Control by uracil of carbamyl phosphate synthesis in *Escherichia coli. Biochim. Biophys. Acta* **69**, 355–360.

Gouesbet G., Jebbar M., Bonnassie S., Hugouvieux-Cotte-Pattat N., Himdi-Kabbab S. and Blanco C. 1995 *Erwinia chrysanthemi* at high osmolarity: influence of osmoprotectants on growth and pectate lyase production. *Microbiology* **141**, 1407–1412.

Gowrishankar J. 1985 Identification of osmoresponsive genes in *Escherichia coli:* evidence for participation of potassium and proline transport systems in osmoregulation. *J. Bacteriol.* **164**, 434–445.

Gowrishankar J. and Pittard J. 1982 Construction from Mu d1 (*lac* Ap^r) lysogens of lambda bacteriophage bearing promoter–*lac* fusions: isolation of λ *ppheA-lac. J. Bacteriol.* **150**, 1122–1129.

Greenway H. and Munns R. 1980 Mechanisms of salt tolerance in nonhalophytes. *Annu. Rev. Plant Physiol.* **31**, 149–190.

Guilloton M. and Karst F. 1987 Cyanate specifically inhibits arginine biosynthesis in *Escherichia coli* K-12: a case of by-product inhibition? *J. Gen. Microbiol.* **133**, 655–665.

Gutierrez C., Barondess J., Manoil C. and Beckwith J. 1987 The use of transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus: analysis of osmotically regulated genes in *Escherichia coli. J. Mol. Biol.* **195**, 289–297.

Haas D., Kurer V. and Leisinger T. 1972 *N*-Acetylglutamate synthetase of *Pseudomonas aeruginosa.* An assay *in vitro* and feedback inhibition by arginine. *Eur. J. Biochem.* **31**, 290–295.

Hafner E. W., Tabor C. W. and Tabor H. 1977 Isolation of a *metK* mutant with a temperature-sensitive *S*-adenosylmethionine synthetase. *J. Bacteriol.* **132**, 832–840.

Harris C. L. 1981 Cysteine and growth inhibition of *Escherichia coli:* threonine deaminase as the target enzyme. *J. Bacteriol.* **145**, 1031–1035.

Hershkovitz N., Oren A., Post A. and Cohen Y. 1991 Induction of water-stress proteins in cyanobacteria exposed to matric- or osmotic-water stress. *FEMS Microbiol. Lett.* **83**, 169–172.

Houssin C., Eynard N., Shechter E. and Ghazi A. 1991 Effect of osmotic pressure on membrane energy-linked functions in *Escherichia coli. Biochim. Biophys. Acta* **1056**, 76–84.

Hunter J. S. V., Greene R. C. and Su C.-H. 1975 Genetic characterization of the *metK* locus in *Escherichia coli* K-12. *J. Bacteriol.* **122**, 1144–1152.

Jensen K. F. 1993 The *Escherichia coli* K-12 "wild-types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. *J. Bacteriol.* **175**, 3401–3407.

Karpel R., Alon T., Glaser G., Schuldiner S. and Padan E. 1991 Expression of a sodium proton antiporter (NhaA) in *Escherichia coli* is induced by Na^+ and Li^+ ions. *J. Biol. Chem.* **266**, 21753–21759.

Käpyaho K. and Jänne J. 1982 Regulation of putrescine metabolism in Ehrlich ascites carcinoma cells exposed to hypotonic medium. *Biochim. Biophys. Acta* **714**, 93–100.

Kelker N. and Eckhardt T. 1977 Regulation of *argA* operon expression in *Escherichia coli* K-12: cell-free synthesis of β-galactosidase under *argA* control. *J. Bacteriol.* **132**, 67–72.

Kunst F. and Rapoport G. 1995 Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis. J. Bacteriol.* **177**, 2403–2407.

Laimins L. A., Rhoads D. B. and Epstein W. 1981 Osmotic control of *kdp* operon expression in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **78**, 464–468.

Leikin S., Parsegian V. A., Rau D. C. and Rand R. P. 1993 Hydration forces. *Annu. Rev. Phys. Chem.* **44**, 369–395.

Leisinger T. and Haas D. 1975 *N*-Acetylglutamate synthase of *Escherichia coli:* regulation of synthesis and activity by arginine. *J. Biol. Chem.* **250**, 1690–1693.

Le Rudulier D. and Bouillard L. 1983 Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the Enterobacteriaceae. *Appl. Environ. Microbiol.* **46**, 152–159.

Le Rudulier D., Yang S. S. and Csonka L. N. 1982 Nitrogen fixation in *Klebsiella pneumoniae* during osmotic stress: effects of exogenous proline or a proline overproducing plasmid. *Biochim. Biophys. Acta* **719**, 273–283.

Le Rudulier D., Strom A. R., Dandekar A. M., Smith L. T. and Valentine R. C. 1984 Molecular biology of osmoregulation. *Science* **224**, 1064–1068.

Mager J. 1955 Influence of osmotic pressure on the polyamine requirement of *Neisseria perflava* and *Pasteurella tularensis* for growth in defined media. *Nature* **176**, 933–934.

Miller J. H. 1992 *A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria.* Cold Spring Harbor Laboratory Press, New York.

Morris D. R. and Koffron K. L. 1969 Putrescine biosynthesis in *Escherichia coli. J. Biol. Chem.* **244**, 6094–6099.

Munro G. F. and Bell C. A. 1973 Polyamine requirements of a conditional polyamine auxotroph of *Escherichia coli. J. Bacteriol.* **115**, 469–475.

Munro G. F. and Sauerbier W. 1973 Osmotically induced excretion of putrescine by mutants of *Escherichia coli* defective in potassium transport. *J. Bacteriol.* **116**, 488–490.

Munro G. F., Hercules K., Morgan J. and Sauerbier W. 1972 Dependence of the putrescine content of *Escherichia coli* on the osmotic strength of the medium. *J. Biol. Chem.* **247**, 1272–1280.

Munro G. F., Miller R. A., Bell C. A. and Verderber E. L. 1975 Effects of external osmolarity on polyamine metabolism in HeLa cells. *Biochim. Biophys. Acta* **411**, 263–281.

Parsegian V. A., Rand R. P. and Rau D. C. 1995 Macromolecules and water: probing with osmotic stress. *Meth. Enzymol.* **259**, 43–94.

Perry J. W. and Oka T. 1980 Regulation of ornithine decarboxylase in cultured mouse mammary gland by the osmolarity in the cellular environment. *Biochim. Biophys. Acta* **629**, 24–35.

Piérard A., Glansdorff N., Mergeay M. and Wiame J. M. 1965 Control of the biosynthesis of carbamoyl phosphate in *Escherichia coli. J. Mol. Biol.* **14**, 23–36.

Posas F., Camps M. and Arino J. 1995 The PPZ protein phosphatases are important determinants of salt tolerance in yeast cells. *J. Biol. Chem.* **270**, 13036–13041.

Poulin R., Wechter R. S. and Pegg A. E. 1991 An early enlargement of the putrescine pool is required for growth in L1210 mouse leukemia cells under hypoosmotic stress. *J. Biol. Chem.* **266**, 6142–6151.

Rubenstein K. E., Streibel E., Massey S., Lapi L. and Cohen S. S. 1972 Polyamine metabolism in potassium-deficient bacteria. *J. Bacteriol.* **112**, 1213–1221.

Sambrook J., Fritsch E. F. and Maniatis T. 1989 *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, New York.

Saroja G. N. and Gowrishankar J. 1996 Roles of SpoT and FNR in NH_4^+ assimilation and osmoregulation in GOGAT (glutamate synthase)-deficient mutants of *Escherichia coli. J. Bacteriol.* **178**, 4105–4114.

Shortridge V. D., Lazdunski A. and Vasil M. L. 1992 Osmoprotectants and phosphate regulate expression of phospholipase C in *Pseudomonas aeruginosa. Mol. Microbiol.* **6**, 863–871.

Singer M., Baker T. A., Schnitzler G., Deischel S. M., Goel M., Dove W., Jaacks K. J., Grossman A. D., Erickson J. W. and Gross C. A. 1989 A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli. Microbiol. Rev.* **53**, 1–24.

Sorensen M. and Pedersen S. 1991 Cysteine, even in low concentrations, induces transient amino acid starvation in *Escherichia coli. J. Bacteriol.* **173**, 5244–5246.

Sutherland L., Cairney J., Elmore M. J., Booth I. R. and Higgins C. F. 1986 Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. *J. Bacteriol.* **168**, 805–814.

Uchida S., Garcia-Perez A., Murphy H. and Burg M. B. 1989 Signal for induction of aldose reductase in renal medullary cells by high external NaCl. *Am. J. Physiol.* **256**, C614–C620.

Wyn Jones R. G. 1984 Phytochemical aspects of osmotic adaptation. *Recent Adv. Phytochem.* **18**, 55–78.

Yancey P. H., Clark M. E., Hand S. C., Bowlus R. D. and Somero G. N. 1982 Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.

Received 19 March 1998