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

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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, K₂SO₄), 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.


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, K₂SO₄ 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

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 glyceral (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 channeled 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](https://example.com/figure1.png)

**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.
Anhydrotic stress in E. coli

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>CAG18475</td>
<td>metC162::TsEl0</td>
<td>Singer et al. 1989</td>
</tr>
<tr>
<td>CAG18559</td>
<td>supG3I157::Tn10Kan</td>
<td>Singer et al. 1989</td>
</tr>
<tr>
<td>CSH141</td>
<td>Δ(gpt-lac)5 supE rpsE/[F lac+ pro+]</td>
<td>Miller 1992</td>
</tr>
<tr>
<td>MA5</td>
<td>gal argA</td>
<td>Celis 1977</td>
</tr>
<tr>
<td>MA255</td>
<td>thr-1 leuB6 can-1 speB2 speC3 thi-1 relA17 lacY1 gal-6 xyl-7 mil-2 rpsL33 tonA2 supE44</td>
<td>Cunningham-</td>
</tr>
<tr>
<td>MC4100</td>
<td>Δ(argF-lac)U169 rpsL150 araD139 relA1 fbbS530l deoC1 ptaF25 rbsR</td>
<td>Rundles and Maas 1975</td>
</tr>
<tr>
<td>MG1655</td>
<td>wild type</td>
<td>Gowrishankar 1985</td>
</tr>
<tr>
<td>PL8-31</td>
<td>thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 supE44 galK2 hisC3 rfdD1 metG87 serA25 metK86 Δ(speC-glc)63 rpsL25 kgkK31 xylA5 mil-1 thi-1</td>
<td>Hunter et al. 1975</td>
</tr>
<tr>
<td>GJ134</td>
<td>MC4100 Δ_purA101 proP222 Δ(pyr-76::Tn10)461</td>
<td>Gowrishankar 1985</td>
</tr>
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</tr>
<tr>
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<td>This study</td>
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<tr>
<td>GJ1201T</td>
<td>GJ134 argA202 zga-901::Tn10dTet</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>GJ1293</td>
<td>MC4100 speC3</td>
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</tr>
<tr>
<td>GJ1294</td>
<td>PL8-31 metC162::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>GJ1296</td>
<td>MC4100 Δ(speC-glc)63</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Gene designations are as described in Berlyn et al. 1996. All strains are F+ unless otherwise indicated. The argR64, recD1901::Tn10 and zha-901::Tn10dKan mutations were sourced from strains MA1030 (Coli Genetic Stock Center), CAG12135 (Singer et al. 1989) and G912 (Saroja and Gowrishankar 1996) respectively. The ΔspecC mutation in PL8-31 was earlier called gic-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 Tn10dTet and Tn10dKan 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 Tn10ΔKan or Tn10ΔTet 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 PI lysates prepared on the pools of Kanr and Test 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. PI lysates prepared on individual colonies purified from these plates were then used to determine linkage between each Kanr or Tetr insertion and the mutation conferring NaCl tolerance in GJ1201. In this manner, one Kanr insertion and one Tetr 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::Tn10ΔKan and zga-901::Tn10ΔTet 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+ transducing of GJ1274 was designated GJ1275.

**Construction of isogenic spec and spec+ strains**: In preliminary transduction experiments into the spec spec (putrescine-auxotrophic) strain MA255, we established that the metC162::Tn10 (in CAG18475) and nupG3157::Tn10ΔKan (in CAG18559) insertions are, respectively, 4% and > 99% linked to spec. A nupG3157::Tn10ΔKan derivative of MC4100 was used as recipient in two transductions to Tet+; the donors were GJ1276 and GJ1294, which had metC::Tn10 linked to spec3 and Δspec respectively. The Tet+ colonies from each cross were scored for Kanr, 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::Tn10 marker was then crossed out from the spec+ mutant strains by transduction to Met+. The resultant strains GJ1293 (spec3) and GJ1296 (Δspec) are thus isogenic derivatives of MC4100 (specC+).

**Other genetic techniques**: The methods for transformation (Sambrook et al. 1989), nitrosoguanidine mutagenesis (Miller 1992), and transduction with phage PI (Govrishankar 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::Tn10 and zha-901::Tn10ΔKan 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 (Govrishankar 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; MgCl2, 10 mM; acetyl CoA, 4.6 mM; L-[1-14C]-glutamic acid (Bhabha Atomic Research Centre, Mumbai) [pH adjusted to 9 with KOH and specific activity adjusted to 0.4 μCi (1.5 × 106 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
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' and Kan' 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::Tnl0 insertion (data not shown).

A derivative of MC4100 (designated GJ1217) carrying the mutation from GJ1201 was constructed by transduction with the linked recD::Tnl0 marker. Strain MC4100 is an ancestor of GJ134 (the latter having additional mutations in putP4, 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 (Pierard 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 betR.) 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Specific activity*</th>
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<tbody>
<tr>
<td>GJ1205</td>
<td>argA4 argR+</td>
<td>1958</td>
</tr>
<tr>
<td>GJ1206</td>
<td>argA202 argR+</td>
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<td>9403</td>
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<tr>
<td>GJ1264</td>
<td>argA202 argR64</td>
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</table>

*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 bradyprotrophic mutation in argA, which we have designated argA202.

(i) In comparison with its isogenic argA4 derivative GJ1205, the argA202 mutant GJ1206 was hypersensitive to the l-arginine analogue l-cavanamide (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 (Welker 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::Tn10 is responsible for the phenotype of uracil–NaCl tolerance, l-cavanamide 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 bradyprotrophic 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 argR64 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 exogenous 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
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**Figure 4.** Growth of MC4100 (*speC*<sup>+</sup>) 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 (Spm). 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 *speC* mutant strain (GJ1293) with that of its isogenic *speC*<sup>+</sup> parent MC4100. Both strains grew equally well on NaCl-supplemented glucose–minimal A–uracil medium (compare top and bottom rows of the extreme left panel in figure 4), and also on uracil-supplemented 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*<sup>+</sup> 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 *speC* 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 *speC* 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 *speC* 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*<sup>+</sup> 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 equimolar 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<sub>2</sub>SO<sub>4</sub> (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 osmoresponsive *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 osmo-regulation (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 anhydrotic 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
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1996), and for argR mutations (through competition for L-ornithine from the enormously derepressed ornithine carbamoyltransferase isoenzymes encoded by the argF and argl 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; Piéard 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* (Craeel 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 (Craeel 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; Kápyaño 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. Cells and C. A. Gross for strains, K. Igarashi for plasmid pODC, and D. Balanabramanian, L. N. Csont and D. P. Kasbar 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.

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Received 19 March 1998