Comparative analysis of naturally occurring L-amino acid osmolytes and their D-isomers on protection of *Escherichia coli* against environmental stresses

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Adaptation to high salinity and low or high temperature is essential for bacteria to survive. Accumulation of exogenous osmolytes is one of the ways that helps bacteria to survive under such extracellular stress. We have analysed the capability of various L-amino acids and their D-isomers to act as osmolytes and thus enable *Escherichia coli* cells to survive under various stress conditions. *E. coli* cells were grown in the presence or absence of L- and D-proline, alanine, serine and lysine under salt, heat and cold stresses. Of the various amino acids tested, L-proline, closely followed by L-serine turned out to be highly protective against environmental stresses. L-proline provided excellent protection (95%) against salt stress, followed by cold (60%) and heat (40%) stresses. D-amino acids on the other hand, proved to be highly inhibitory under stress conditions. Thus L-amino acids were found to be growth protectants under stress while their D-isomers were inhibitory during stress as well as normal conditions.

[Shahjee H M, Banerjee K and Ahmad F 2002 Comparative analysis of naturally occurring L-amino acid osmolytes and their D-isomers on protection of *Escherichia coli* against environmental stresses; *J. Biosci*. **27** 515–520]

1. Introduction

Extremes of medium osmolarity are generally deleterious to cell growth. The ability of organisms to adapt to osmotic stress is a fundamental biological process that protects them against fluctuations in water activity and solute content in their environment (Csonka 1982). Many organisms including bacteria, yeast, plants and animals can adapt to various hyperosmotic stress by accumulating low-molecular-mass organic compounds known collectively as osmolytes (Yancey *et al* 1982). Intracellular accumulation of osmolytes either by *de novo* synthesis or by transport from the growth medium confers tolerance to hyperosmotic stress (Csonka 1982; Gowrishanker 1985; Roth *et al* 1985). It is well established that the intracellular accumulation of these solutes prevents water loss and maintains the turgor pressure of the cell essential for the cell growth. These molecules also stabilize the native state of various globular proteins against denaturing stresses and favour formation of protein assemblies (Baskakov and Bolen 1998; Yancey *et al* 1982). By themselves, these solutes do not perturb the functional activity of macromolecules and hence are compatible with protein function (Booth *et al* 1988; Csonka 1982; Imhoff 1986; Koch 1983).

Stress induced by heat leads to protein denaturation, misfolding and aggregation of proteins (Cohen *et al* 1991). Cold stress, on the other hand, may lead to stabilization of the secondary structure of nucleic acids with ensuing inhibition of DNA replication, gene transcription and mRNA translation. It may also decrease the activity of many enzymes with the consequent slow down of metabolism (De Macario and Macario 2000).

While L-isomers of amino acids are predominantly found in all living organisms, D-amino acids in general

Keywords. *Escherichia coli*; D-amino acids; L-amino acids; osmolyte; stress

are also not uncommon in nature (Corrigan 1969). Cell walls of Gram negative bacteria contain D-alanine, antibiotic peptides contain D-amino acids (Katz and Demain 1977). Many invertebrates have free D-amino acids in their body fluids which are known to participate actively in metabolism (Felbeck 1980; Matsushima *et al* 1984; Schoettler *et al* 1983). D-enantiomers of amino acids have also been detected in human physiological fluids (Ercal *et al* 1996). However, under stressed conditions, living organisms are known to accumulate only L-isomers of amino acids while no D-amino acid has been found to accumulate till date. Why living organisms have evolved to select only L-amino acids as naturally occurring osmolytes is a matter of debate.

Though it has previously been observed that D-amino acids can inhibit the growth of *E. coli* under nonstressed conditions (Trippen *et al* 1976), no comparative studies of L- and D-amino acids during stress have previously been made. The present study was carried out to understand the role of various L- and D-amino acids like lysine, alanine, serine and proline on the protection of bacteria (*E. coli*) against various kinds of environmental factors like salt, heat and cold stress. We observed that L- and D-isomers of these amino acids have totally opposite effects on *E. coli* cell growth. Of the various amino acids used, L-proline followed by L-serine were found to be highly protective under the stress conditions used, thereby raising the possibility of using them for universal stress protection. On the other hand all the D-amino acids had an inhibitory effect on cell growth.

2. Materials and methods

2.1 *Bacterial growth medium*

E. coli (DH5*a*) bacteria was used as an experimental model for these *in vitro* studies. The bacterial cells were grown in minimal medium comprising of – 100 mM KH_2PO_4, 15 mM $(NH_4)_2PO_4$, 0·16 mM $MgSO_4$ and 3·9 M $FeSO_4$. The carbon source was 10 mM D-glucose. Amino acids used were L- and D-proline, L- and D-lysine, L- and D-serine and L- and D-alanine (Sigma-Aldrich, USA). Salt stress was given by NaCl (Merck, India). The minimal medium was freshly prepared and sterilized through a 0·45 μm filter.

2.2 *Bacterial culture*

A single colony was inoculated into minimal medium and grown overnight. This primary culture was distributed into equal aliquots and frozen at – 70°C. For each experiment, one such aliquot was used to grow an overnight culture. Equal quantity of this overnight culture was used as innoculum for each experiment to ensure equal innoculum of viable bacteria.

2.3 *Induction of stress*

For the salt stress experiments, cells were grown overnight at 37°C in the presence and absence of salt (0·1–0·9 M) with or without L- and D-amino acids. For the temperature stress experiments, *E. coli* cells were grown overnight at different temperatures (15, 25, 37, 45 and 55°C) in the presence or absence of various L- and D-amino acids. Cell growth was monitored by taking absorbance of cells in a UV/Vis spectrophotometer at 578 nm.

3. Results

3.1 *Effect of salt stress on growth at 37°C*

E. coli (DH5*a*) cells were grown overnight at 37°C in the presence of NaCl concentration ranging from 0·1 M to 0·9 M NaCl. Cells grow at normal rates up to 0·2 M NaCl. Higher concentrations of NaCl inhibited the growth of cells (figure 1a). Since 50% inhibition of growth was achieved at about 0·9 M, this concentration of NaCl was used to induce salt stress in further experiments.

3.2 *Effect of L-amino acids on growth under salt stress at 37°C*

In order to find out the protective efficacy of L-amino acids on cell growth under salt stress, *E. coli* was grown under various concentrations of L-amino acids in the presence of 0·9 M salt. In these studies, maximum growth enhancement was observed with L-proline (95% over control) at 15 mM, followed by L-serine (85%) at 15 mM, L-alanine (70%) at 10 mM and least by L-lysine (60%) at 10 mM (figure 1b).

3.3 *Comparative analysis of effect of L- and D-isomers under salt stress at 37°C*

Comparative studies were carried out for monitoring the growth of *E. coli* in the presence of L- and D-amino acids, under salt stress. The concentrations of D-isomers used for bacterial growth were the same at which maximal stimulation was observed with L-isomers (see figure 1b). It was found that unlike L-isomers all D-amino acids further inhibited the growth of *E. coli* cells under salt stress (figure 1c).

3.4 *Effect of L- and D-amino acids on growth of* E. coli *under cold stress*

In order to see if the L- and D-isomers behave in a similar fashion at low temperatures, cell growth was measured in the presence or absence of L- and D-amino acids under cold stress given at 15 and 25°C and data was compared with that of control cells grown at 37, 15 and 25°C in the absence of amino acids. It was observed (figure 2) that L-amino acids, particularly L-proline and

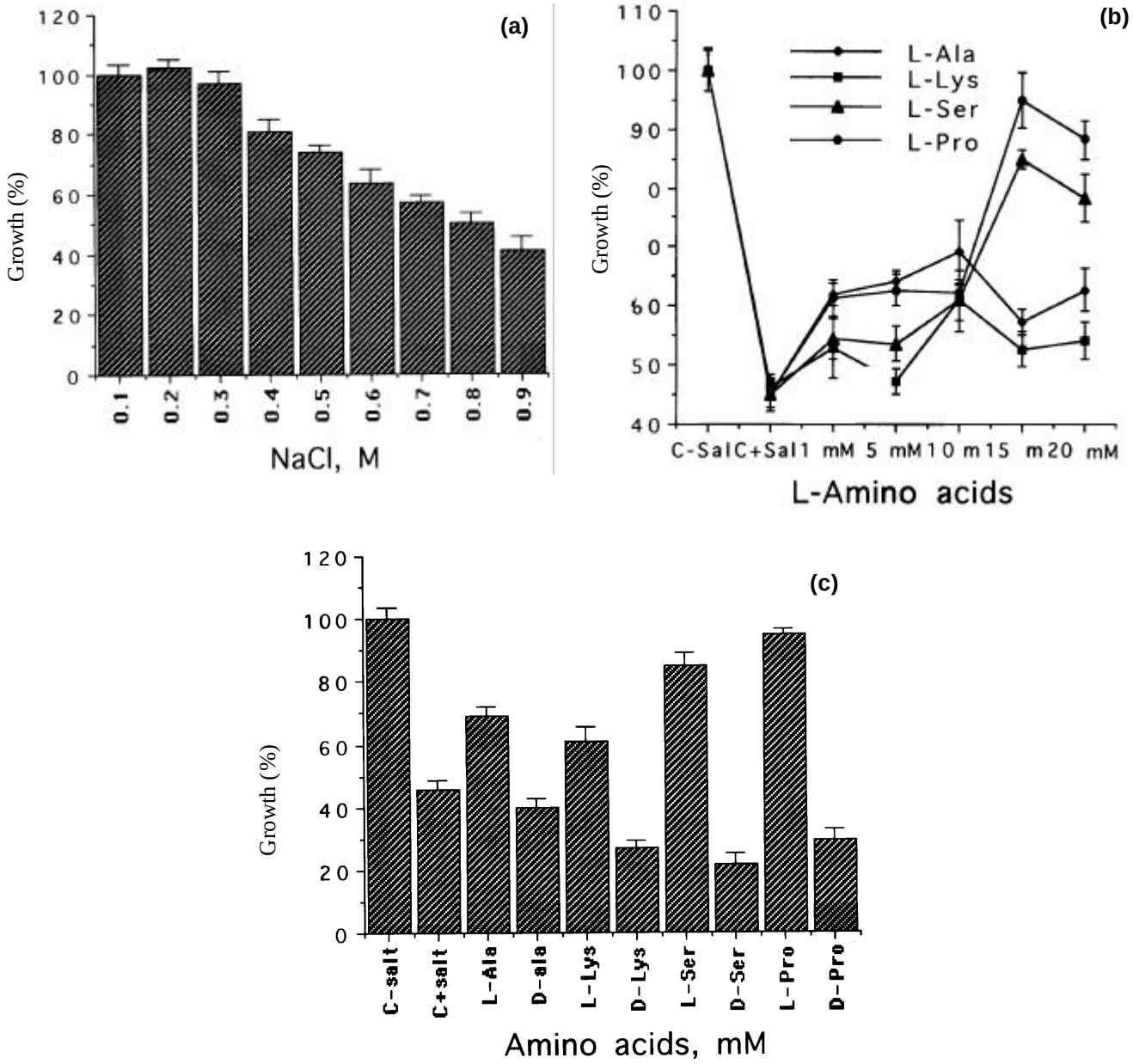

Figure 1. (**a**) Effect of salt stress on the growth of *E. coli*. *E. coli* cells were grown in the presence of various concentrations of NaCl ranging from 0·1 to 0·9 M. The growth of cells was plotted as percent growth relative to control cells grown in the absence of any stress (taken as 100%). (**b**) Effect of increasing concentrations of L-amino acids on the growth of *E. coli*. *E. coli* cells were grown in the presence of various concentrations in the range (1–20 mM) of L-alanine, L-lysine, L-serine and L-proline in the presence of 0·9 M NaCl. The growth of cells was plotted as percent growth relative to control cells (taken as 100%) grown in the absence of salt and osmolytes as a function of each amino acid concentration. (**c**) Effect of L- and D-amino acids on growth of *E. coli* during salt stress. *E. coli* cells were grown in the presence of 15 mM L- or D-Ser, 15 mM L- or D-Pro, 10 mM L- or D-Ala and 10 mM L- or D-Lys in the presence of 0·9 M NaCl. The growth of cells was plotted as percent growth relative to control cells (taken as 100%) grown in the absence of salt and amino acids and compared with control cells with salt but without amino acids.

L-serine were able to protect the cells from the inhibitory effects of cold stress and D-amino acids were again found to be inhibitory. These studies clearly indicate that L-isomers of amino acids can protect the cells from cold stress.

3.5 *Effect of L- and D-amino acids on growth of* E. coli *under heat stress*

Studies on the growth of *E. coli* were carried out in the presence or absence of L- and D-amino acids at higher temperatures, namely, 45 and 55°C (figure 3). Results of these studies were compared with that of control cells grown at 37, 45 and 55°C in the absence of amino acids. It was seen that the growth of cells was slightly protected from the inhibitory effects of heat stress in the presence of L-amino acids whereas D-amino acids acted antagonistically.

4. Discussion

Under stress conditions, particularly osmotic dehydration, *E. coli* has been seen to accumulate L-amino acids (Yancey *et al* 1982; Imhoff 1986). In comparison, even though D-amino acids have been found to be abundantly present in animals (Corrigan 1969), their role during different types of stress has not been clearly established. In the present study a comparative analysis of various L-amino acids and their D-enantiomers was done to explore their role as a protective osmolyte against various kinds of environmental stress. Non-halophillic bacteria differ greatly in their tolerance to NaCl in their growth medium (Measures 1975). In this study, *E. coli* (DH5*a*) cells grew at a normal rate up to 0·2 M NaCl, higher concentrations resulted in decreased cell growth and at 0·9 M almost 50% inhibition of growth occurred (see figure 1a). This implied that the mechanism of cellular responses to elevated osmotic strength was incomplete or that it impaired the efficiency of metabolic processes. An exogenous supply of osmoprotectant amino acids such as L-proline, glycine–betaine has been shown to enhance the ability of the cell to grow in a hyper-osmotic medium (Csonka 1982; Gowrishanker 1985; Measures 1975; Roth *et al* 1985).

In our previous *in vitro* studies (unpublished data) regarding the effect of various L- and D-amino acids on protein conformation, it was observed that proline, serine, lysine, and alanine had comparable effects on protein

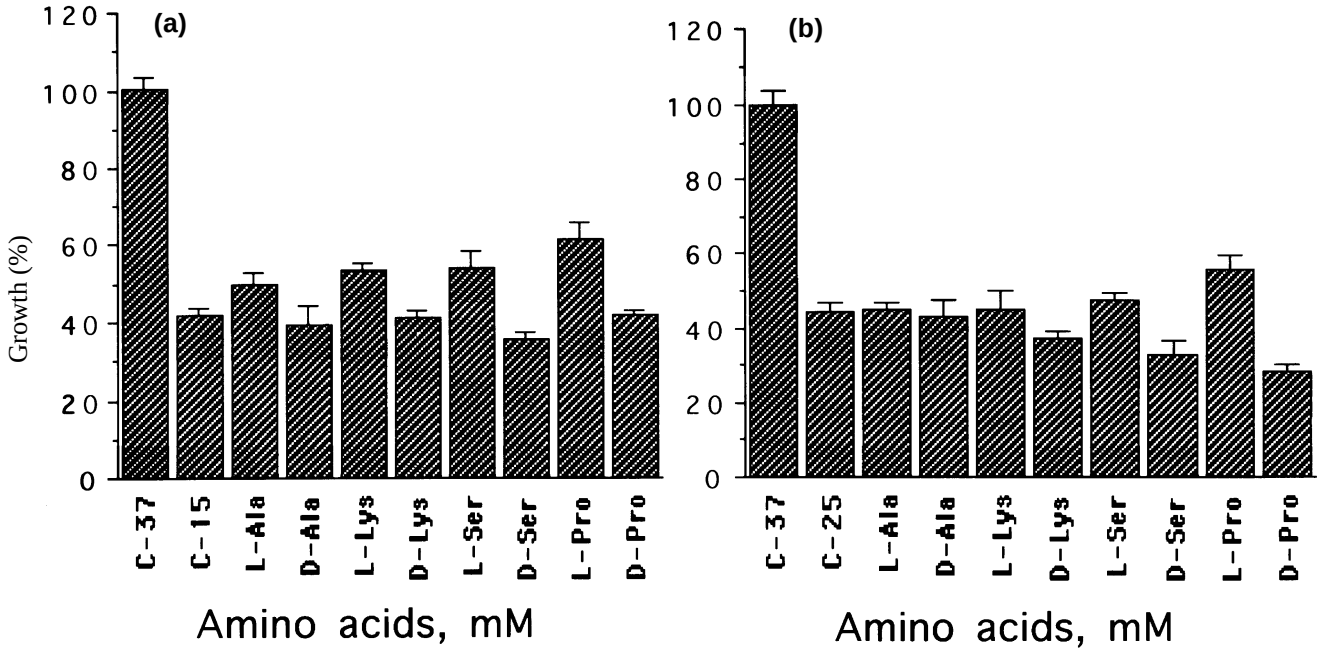

Figure 2. (**a**) Effect of L- and D-amino acids on growth of *E. coli* during cold stress (15°C). *E. coli* cells were grown in the presence of 15 mM L- or D-Ser, 15 mM L- or D-Pro, 10 mM L- or D-Ala and 10 mM L- or D-Lys at 15°C. The growth of cells was plotted as percent growth relative to control cells grown at 37°C (taken as 100%) without amino acids and compared with control cells grown at 15°C without amino acids. (**b**) Effect of L- and D-amino acids on growth of *E. coli* during cold stress (25°C). *E. coli* cells were grown in the presence of 15 mM L- or D-Ser, 15 mM L- or D-Pro, 10 mM L- or D-Ala and 10 mM L- or D-Lys at 25°C. The growth of cells was plotted as percent growth relative to control cells grown at 37°C (taken as 100%) without amino acids and compared with control cells grown at 25°C without amino acids.

stability and activity. It was established from those studies that both these isomers of amino acids had higher stabilizing effects on the proteins against thermal denaturation and also did not perturb enzyme activity at room temperature. Therefore all four amino acids were selected for this *in vivo* study. Though it has been previously observed that the growth rate of osmotically stressed cells is directly related to the amount of osmoprotectant accumulated (Cayley *et al* 1992), we observed that bacterial cells showed dose dependent growth stimulation with all the L-amino acids, reached a peak at 10 mM (L-alanine and L-lysine) or 15 mM (L-serine and L-proline) (see figure 1b) but declined thereafter.

It has been reported that exogenously supplied L-pipecolic acid (an imino acid) helped in growth restoration of *E. coli* cells under hyperosmolarity whereas D-isomer of this imino acid had an inhibitory effect on growth *of E. coli* cell (Gouesbet *et al* 1994). Among various L- and D-amino acids tested, we found that L-amino acids were able to protect the cells against salt stress (figure 1c). In the absence of stress (i.e. cells grown at 37°C without salt) D-amino acids generally showed very little inhibition of cell growth (data not shown). On the other hand, under hyperosmotic stress, there was no protection instead there was substantial inhibition of cell growth (figure 1c).

Our results suggest that only L-amino acids and not their D-isomers, act as osmoprotectants. The *in vivo* inhibition caused by D-isomers is surprising, as it has previously been proposed that D-isomers of amino acids should be more compatible than L-isomers as they are least interacting with enzymes (Somero 1986). The inhibition caused by D-amino acids may be due to the fact that the cells are unable to recognize the D-form of amino acids, accumulated or transported from the media. With increasing salinity the effect maybe more pronounced due to the activation of uptake systems that may cause leakage of D-isomers into the cytoplasm. Further, these bacteria may lack D-amino acid oxidase and D-aspartate oxidase; two enzymes which can metabolize D-amino acids and prevent the cell from any inhibitory effect (D'Aniello 1993). It is also possible that the D-amino acids in the cell get concentrated to the extent that these enzymes are unable to metabolize them which might cause serious damage to the cell by suppressing synthesis of various other enzymes (D'Aniello 1993). Presently there are no reports of any transport mechanism of D-amino acids.

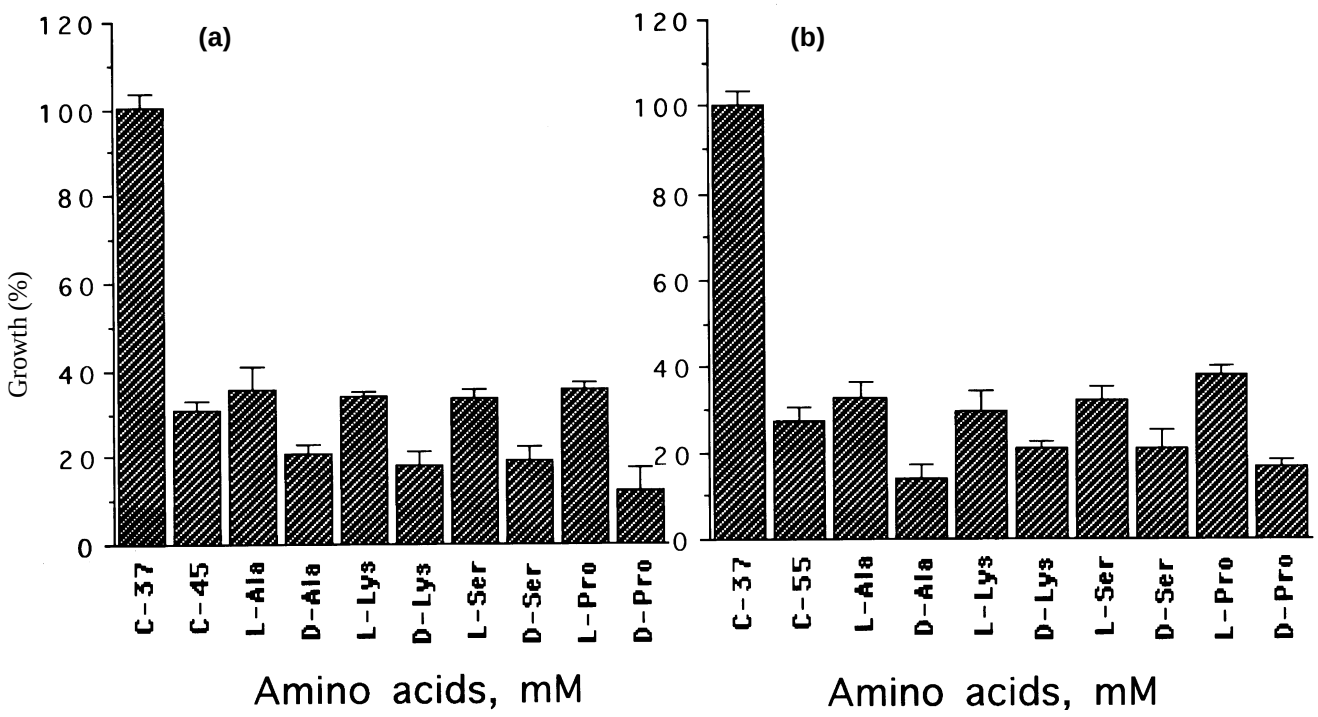

Figure 3. (**a**) Effect of L- and D-amino acids on growth of *E. coli* during heat stress (45°C). *E. coli* cells were grown in the presence of 15 mM L- or D-Ser, 15 mM L- or D-Pro, 10 mM L- or D-Ala and 10 mM L- or D-Lys at 45°C. The growth of cells was plotted as percent growth relative to control cells grown at 37°C (taken as 100%) without amino acids and compared with control cells grown at 45°C without amino acids. (**b**) Effect of L- and D-amino acids on growth of *E. coli* during heat stress (55°C). *E. coli* cells were grown in the presence of 15 mM L- or D-Ser, 15 mM L- or D-Pro, 10 mM L- or D-Ala and 10 mM L- or D-Lys at 55°C. The growth of cells was plotted as percent growth relative to control cells grown at 37°C (taken as 100%) without amino acids and compared with control cells grown at 55°C without amino acids.

L-amino acids particularly L-proline, were found to be protective against heat and cold stresses. This effect was more pronounced during cold stress where the cells showed better growth (60%) in the presence of L-proline compared to cells grown without osmolytes (40%) (figure 2). L-amino acids were able to marginally (35–40%) rescue cells during heat stress (30%) (see figure 3). This difference in level of protection by osmolytes during salt and temperature stress could be due to the fact that osmolytes are accumulated better during salt stress as compared to temperature stress. A similar study had recently shown that glycine–betaine was able to protect cells when both salt and heat stress were given together; glycine–betaine accumulated due to salt stress could protect against heat stress also (Diamant *et al* 2001)

On the other hand, D-amino acids were once again found to be inhibitory during heat as well as cold stress. To the best of our knowledge this is the first report where it has been shown that L-amino acids but not their D-enantiomers can protect cell growth against other kinds of stress like temperature stress.

In conclusion we have found that L-amino acids particularly L-proline, are excellent protective osmolytes that not only protect *E. coli* cells from osmotic stress but also from other environmental stresses like cold and heat. Surprisingly, D-isomers of the same amino acids prove to be highly inhibitory under stress conditions *in vivo*, even though they have no negative effect on protein stability and activity *in vitro*. Future studies can reveal the specific stress proteins that are upregulated in the bacteria by L-amino acids but not by their D-isomers.

Acknowledgements

We thank Dr Seyed E Hasnain for helpful suggestions and comments on this manuscript. FA would like to acknowledge the financial support from the Council of Scientific and Industrial Research and Department of Science and Technology, New Delhi.

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MS received 21 March 2002; accepted 8 May 2002

Corresponding editor: SEYED E HASNAIN