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Proline Over-Production Results in Enhanced Osmotolerance in *Salmonella typhimurium*

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Summary. Mutants of *S. typhimurium* with enhanced osmotolerance were isolated. These mutants were obtained as strains which over-produced proline due to regulatory mutations affecting proline biosynthesis. The mutations are located on F'*proBA* and upon transfer to other *S. typhimurium* strains, they confer enhanced osmotolerance on the recipients. The osmotolerant mutants not only have higher intracellular proline levels than the osmosensitive parental strain, but the proline levels in the osmotolerant mutants are regulated such that they increase in response to osmotic stress. Possible reasons why elevated proline levels lead to enhanced osmotolerance are discussed.

The osmotic strength of the environment is one of the important parameters determining the ability of organisms to thrive in a given habitat. A variety of strategies have evolved for the regulation of the internal osmolarity of organism but the details of these osmoregulatory mechanisms are poorly understood. In bacteria internal osmolarity is maintained mainly by the accumulation of inorganic ions and amino acids, such that it exceeds the osmolarity of the growth medium (Christian and Waltho 1961; Tempest et al. 1970; Brown and Stanley 1972; Measures 1975). A discovery made a quarter century ago by Christian (1955a and b) suggested that proline has a special role in osmoregulation: the growth rate and oxygen consumption of *Salmonella orianenburg* were stimulated specifically by the addition of proline in media of inhibitory osmotic strength. In order to investigate this phenomenon further, and to test whether it could be exploited for devising a general selection scheme for osmotolerant organisms, we isolated proline over-producing mutants of *Salmonella typhimurium*, some of which proved to have acquired a growth advantage in media of elevated osmolarity.

Materials and Methods

Culture Conditions. Minimal medium used was Medium 63 (Cohen and Rickenberg 1956) consisting of 0.1 M KH_2PO_4, 0.075 M KOH, 0.015 M $(NH_4)_2SO_4$, 1.6×10^{-4} M $MgSO_4\cdot7H_2O$, and 3.9×10^{-6} M $FeSO_4\cdot7H_2O$. D-glucose, 10 mM (unless otherwise stated) was the carbon source. When required, L-amino acids were added at 0.2 mM. Solid media contained an additional 20 g agar (Difco) per liter. The osmotic strength of the medium was increased by the addition of sucrose or NaCl, as indicated. In the case of NaCl, it was necessary to readjust the pH to that of the normal Medium 63 (pH=7.2) which was done by the addition of NaOH in amounts less than 10% of the NaCl used. Cultures were grown aerobically at 37° C.

Bacterial Strains. Salmonella typhimurium strains TL1 (wild type LT2), JL2468 (del(*proBA*)-*47 leuD798 argI537 ara-9 fol-1*/F'_{128} *proB*$^+$*A*$^+$ *argF*$^+$ *lacI*^{q}::*Z*$^+$*Y*$^+$*A*$^+$) and TR3290 (del(*proBA*)-*47* del(*trp*)-*130 argI539 met E338 amtA1*) were obtained from J. Ingraham. TL117 (del(*proBA*)-*47*) was constructed by transducing TL1 to 8-azaguanine (300 μg/ml) resistance (Sanderson and Hartman 1978) by phage P22 grown on ProAB47 (del(*proBA*)-*47*) obtained from J. Roth. TL106 (*putA842*::Tn*5*) was constructed by transducing TL1 to kanamycin sulfate (75 μg/ml) resistance by P22 grown on TT2601 (*putA842*::Tn*5*) provided by J. Roth. Strains TL81 (F'*pro-75*), TL82 (F'*pro-76*), TL85 (F'*pro-78*), TL86 (F'*pro-73*), TL87 (F'*pro-79*), and TL88 (F'*pro-74*) are L-azetidine-2-carboxylate resistant derivatives of strain JL2468 (see Results). Strains TL119, TL120, TL123, TL124, TL125, TL126, and TL128 are prototrophic progeny of mating strain TL117 with strains TL81, TL82, TL85, TL86, TL87, TL88, and JL2468, respectively. The generalized transducing phage used in all transductions was P22 HT104/1 int201 (from J. Roth), and transdructions were performed as described (Hoppe et al. 1979).

Measurements of the Intracellular Amino Acid Levels. In order to reduce the size of the NH_4^+ peak during amino acid analysis, for this experiment the $(NH_4)_2SO_4$ concentration of Medium 63 was reduced to 0.0075 M and K_2SO_4 was included at a final conentration of 0.0075 M. The strains to be tested were grown in this medium with 20 mM glucose and the indicated concentration of NaCl for at least 4 generations, to an approximate density of 5×10^8 cells/ml. Then 4 ml of the cultures were rapidly filtered on Millipore filters (9 cm diameter 0.45 μm pore size). The filters were placed into 10 ml of 70% (v/v) ethanol containing 100 nmoles of D-norleucine, and the cells were extracted at room temperature for 20 min, with occasional gentle agitation (Pulman and Johnson 1978). The cell debris was removed by centrifugation, and the ethanol extract evaporated to dryness at 60° under a stream of N_2. The residue was taken up in 0.2 M sodium citrate (pH 2.2) and aliquots rund on the Dionix 500 amino acid analyzer. The amounts of amino acids present in the original extract were calculated on the basis of recovery D-norleucine. The results are expressed as nmoles of each amino acid per mg of cellular protein, with the latter determined by the method of Lowry et al. (1951), using lysates of parallel samples of the original cultures. The glutamine peak eluted between the threonine and serine peaks and glutamine was not completely resolved from the other two. We assigned glutamine to the peak so designated because the elution time of the midpoint of the peak agreed with that of a known glutamine standard. Also, when one pilot sample, obtained from strain TL128 grown in 0.65 M NaCl, was hydrolyzed by 6 M HCl in an evaluated tube, the putative glutamine peak disappeared with a corresponding inrease in the glutamate peak.

Results

Proline Stimulates the Growth of Salmonella typhimurium in Media of Inhibitory Osmolarity

Because the motivation for the experiments presented below was provided by the discovery of Christian (1955a and b) that proline

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could alleviate osmotic inhibition in *Salmonella orianenburg*, we wanted to confirm and further characterize this phenomenon with *Salmonella typhimurium*. The basic observation is illustrated in Fig. 1. NaCl, at a concentration of 0.7 M or 0.9 M decreases the growth rate of the organism from 1.0 generation per hour found in the normal medium, to 0.13 and 0.014 generation per hour, respectively. The addition of proline at a concentration of 0.5 mM, stimulates the NaCl inhibited growth, so the growth rates with 0.7 M and 0.9 M NaCl are now 0.29 and 0.11 generation per hour, respectively (Fig. 1A). A similar result is seen in Fig. 1B, where growth is inhibited by 0.8 M sucrose. Proline also has stimulatory effects in the presence of inhibitory concentrations of a number of other solutes, including $(NH_4)_2SO_4$, K_2SO_4, and KH_2PO_4 (data not shown). Proline is unique in this regard because, none of the other 19 common amino acids caused a comparable stimulation (Table 1). (L-cysteine, which is slightly inhibitory to *S. typhimurium* in minimal medium (Filutowicz et al. 1979) is completely inhibitory in the presence of 0.65 M NaCl. The reason for this inhibition is not clear.). Figure 2 shows that the concentrations of proline required for the stimulation are slight in comparison to those of the inhibitory solutes: 0.5 mM proline results in maximal stimulation, and approximately 0.1 mM is sufficient for half maximal effect. Catabolism of proline is not necessary because a *putA*$^-$ mutation, which blocks the only known proline catabolic pathway of the organism (Ratzkin et al. 1978) does not diminish the stimulatory effect (Fig. 2).

The Isolation of Proline Over-Producing Mutants with Increased Osmotolerance

Proline over-producing mutants were selected as strains resistant to a toxic proline analogue, L-azetidine-2-carboxylate, the rationale for the methodology being that proline, produced at high levels, could antagonize the analogue. However, only 1% of the L-azetidine-2-carboxylate resistant mutants proved to be proline over-producers. The same phenotype could also be conferred by a much more frequent type of a mutation, *putP*$^-$, inactivating the major proline permease, which functions in the uptake of the analogue (Ratzkin et al. 1978). Previously, Condamine (1971) isolated mutations resulting in proline over-production, some of which proved to be closely linked to the *proBA* genes, which code for the first and second enzymes of the proline biosynthetic pathway. This fact provided us with a short-cut in the isolation of a large number of additional proline over-producing strains. We have used strain, JL2468 which carried the *proB*$^+$*A*$^+$ genes on a self-transmissible plasmid, F'_{128}, because derivatives which became resistant to L-azetidine-2-carboxylate due to a mutation in the *proBA* region could be readily identified on the basis of their ability to transfer the mutation to other *proB*$^-$*A*$^-$ *Salmonella typhimurium* strains.

For the selection, 2×10^8 cells of JL2468 (del(*proBA*)-*47 leu*$^-$/ F'_{128} *proB*$^+$*A*$^+$) were spread on glucose, leucine, minimal medium supplement with 0.5 mM L-azetidine-2-carboxylate. Colonies resistant to the analogue appeared at the approximate frequency of 1 per 10^5 cells plated. These colonies were printed on a lawn of 2×10^8 cells of TR3290 (del(*proBA*)-*47*) on glucose, tryptophan, methionine minimal plates also supplemented with 0.5 mM L-azetidine-2-carboxylate. One hundred seven (not necessarily independent) derivatives of JL2468 were found which could reproducibly transfer prototrophy and L-azetidine-2-carboxylate resistance to TR3290.

These derivatives of JL2468 were tested for enhanced osmotolerance on solid media containing glucose, leucine and 0.65 M

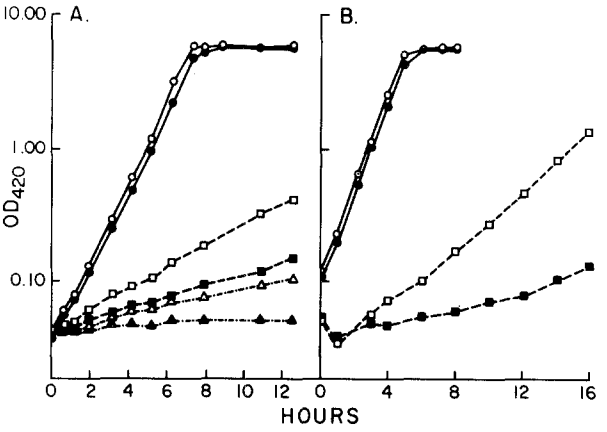

Fig. 1. Stimulation of growth rate of *S. typhimurium* TL1 (wild type) by proline in media of inhibitory osmolarity. The optical density of the cultures is plotted as a function of incubation time. *Closed symbols*: growth without proline; *open symbols*: growth with 0.5 mM proline. Panel A: *circles*: growth in Medium 63; *squares*: growth with an additional 0.7 M NaCl; *triangles*: growth with an additional 0.9 M NaCl. Panel B: *circles*: growth in medium 63; *squares*: growth with an additional 0.8 M sucrose

Table 1. The stimulation of growth rate of *Salmonella typhimurium* TL1 (wild type) by amino acids in the presence of 0.65 M NaCl[a]

Amino Acid	Generation per hour
None	0.20
L-proline	0.40
L-glutamine	0.24
L-serine + glycine	0.23
L-arginine	0.22
L-glutamate	0.22
Other L-amino acids	0.21–0.18
L-cysteine	No growth in 4 days

[a] All 20 common amino acids were added at a final concentration of 0.5 mM to cultures of strain TL1 grown in medium 63 containing 0.65 M NaCl

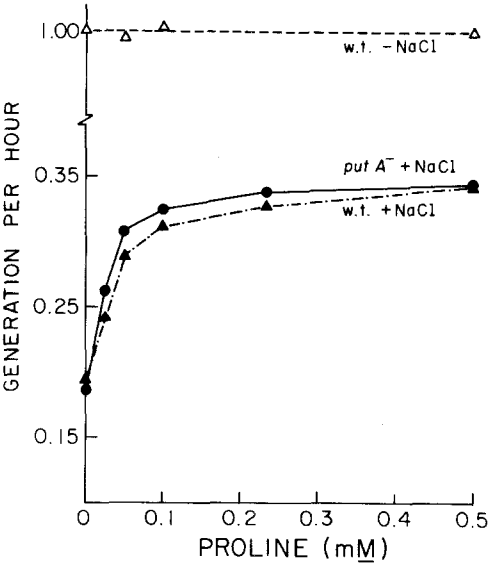

Fig. 2. Stimulation of growth rate by proline in 0.65 M NaCl. Growth rate is plotted as a function of proline concentration of the medium. *Open triangles*: growth rate of TL1 (wild type) in Medium 63; *closed trianges*: growth rate of TL1 in Medium 63 containing 0.65 M NaCl; *closed circles*: growth rate of TL106 (*putA*$^-$) in Medium 63 containing 0.65 M NaCl

NaCl. Of the 107, there were only 6 which after 5 days of incubation were judged to give rise to larger colonies than JL2468. The F′ plasmids, presumably carrying the mutations which caused enhanced osmotolerance, were transferred from these strains into strain TL117 (del(*proBA*)-*47*), and the L-azetidine-2-carboxylate resistance and osmotolerance of the resultant strains were compared to that of TL128, an isogenic strain harboring the original F'_{128} *proB*$^+$*A*$^+$ (Table 2). Although all 6 of the resultant strains carrying the mutant F′ plasmids exhibited similar resistance to L-azetidine-2-carboxylate, only one, strain TL126 was markedly more osmotolerant than the control strain TL128 in the presence of both 0.65 M and 0.8 M NaCl. Four other strains: TL123, TL124, TL119, and TL125 showed slight increases in osmotolerance in the presence of 0.8 M NaCl. The differences between the growth rates of these strains and that of the control strain TL128 were less pronounced in the presence of 0.65 M NaCl. The growth curves of strains TL126 (F′*pro-73*) and TL128 (F′*proB*$^+$*A*$^+$) in the presence of 0.65 M and 0.8 M NaCl are shown in Fig. 3. As was the case with the less osmotolerant strains, the osmotolerant phenotype of strain TL126 was more pronounced under conditions of extreme osmotic inhibition. In 0.65 M NaCl, the growth rates of strains TL126 and TL128 were 0.42 and 0.15 generation/hour, respectively, whereas in 0.8 M NaCl, TL126 had a growth rate of 0.17 generation/hour and strain TL128 did not grow at all. Strain TL126 also had a growth advantage in media whose osmolarity had been increased by high concentrations of sucrose (data not shown). The *pro-73* mutation, which gave rise to the most pronounced osmotolerant phenotype in *Salmonella typhimurium*, was transferred by F′ mediated conjugation to *Escherichia coli* K12 and *Klebsiella pneumoniae* and it conferred similar enhanced osmotolerance on the latter organisms (D. Le Rudulier, S.S. Yang, and L.N. Csonka, mauscript in preparation).

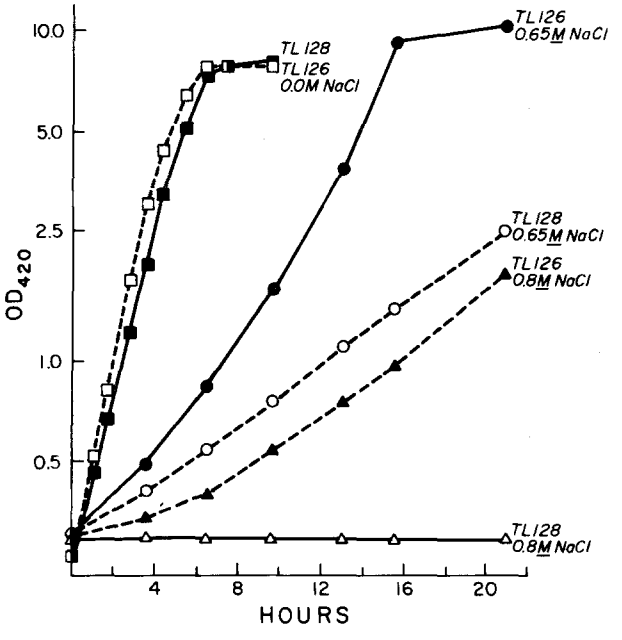

Fig. 3. The effect of the proline over-producing mutation, *pro-74*, on the growth of *S. typhimurium* in media of inhibitory osmolarity. Optical density of the cultures is plotted as a function of incubation time. *Open symbols*: control strain TL128 (F′*proB*$^+$*A*$^+$); *closed symbols*: proline over-producing strain TL126 (F′*pro-74*). *Squares*: growth in Medium 63 with 20 mM glucose; *circles*: growth with an additional 0.65 M NaCl; *trianges*: growth with an additional 0.8 M NaCl

The Amino Acid Levels in the Osmotolerant Strains

In order to determine whether the L-azetidine-2-carboxylate resistant mutants indeed had increased intracellular proline levels, the free amino acid content of cells, grown in minimal medium and medium of elevated osmolarity, was determined. Table 3 summarizes the results obtained with strain TL128: glutamine and glutamic acid were the two most prominent amino acids, and there was a 14- and 2-fold increase in their levels, respectively, upon osmotic stress. Similar results were obtained with other *S. typhimurium* strains, including the L-azetidine-2-carboxylate resistant mutants described above (data not shown), and *E. coli* (Munro et al. 1972). The increases in the glutamine and glutamate levels may be part of the osmoregulatory mechanisms of these organisms.

The proline levels in the L-azetidine-2-carboxylate resistant strains are presented in Table 4. There are three points to be made about these data. First, the mutations originally selected to confer L-azetidine-2-carboxylate resistance result in proline over-production because the proline levels in these strains were

Table 2. The enhanced osmotolerance and L-azetidine-2-carboxylate resistance of the proline over-producing mutants

Strain	Growth rate (generation/h)			
	Minimal medium	Minimal medium +0.65 M NaCl	Minimal medium +0.8 M NaCl	Minimal medium +1 mM L-azetidine-2-carboxylate
Control strain: Tl128 (*pro*$^+$)	1.0	0.15	no growth	0.12
L-azetidine-2-carboxylate resistant derivatives:				
TL126 (*pro-74*)	0.83	0.42	0.17	0.71
TL123 (*pro-78*)	0.77	0.20	0.050	0.77
TL124 (*pro-73*)	0.77	0.18	0.065	0.83
TL119 (*pro-75*)	0.77	0.15	0.042	0.83
TL125 (*pro-79*)	0.83	0.17	0.012	0.83
TL120 (*pro-76*)	0.83	0.16	no growth	0.83

Table 3. The intracellular amino acid levels in the control strain TL128 (F′*proB*$^+$*A*$^+$)

Compound	Intracellular level (nmol/mg protein)	
	Minimal medium	Minimal medium +0.65 M NaCl
Glutamate	136±8	290±53
Glutamine	10±1	142±40
Aspartate	11±3	7±1
Proline	<1.2	<1.9
Other amino acids (total)[a]	11±4	11±4
Ammonia	204±56	332±40

[a] These include glycine, alanine, isoleucine, leucine, and lysine. Other amino acids were undetectable: <1.2 nmol/mg protein in minimal medium; <1.9 nmol/mg protein in 0.65 M NaCl. The results are the averages of two determinations using independent cultures, with the deviation from the mean indicated

Table 4. Proline levels in the proline over-producing mutants

Strain	Proline level (nmol/mg protein)[a]	
	Minimal medium	Minimal + 0.65 M NaCl
TL128[b] (*pro*$^+$)	< 1.2	< 1.9
TL126 (*pro-74*)	36 ± 4	787 ± 100
TL123 (*pro-78*)	19	98
TL124 (*pro-73*)	24 ± 3	75 ± 11
TL119 (*pro-75*)	19	55
TL125 (*pro-79*)	19	22
TL120 (*pro-75*)	4	39

[a] The results for strains TL128, TL126, and TL124 are the averages of 2 independent measurements with the deviation from the mean indicated. The others are the results of one measurement

[b] Trom Table 2

greater than those in strain TL128, both in minimal medium and in the presence of 0.65 M NaCl. Second, in general, the proline levels in these strains were greater when they were grown in 0.65 M NaCl than when they were grown in minimal medium. This effect was most pronounced in strain TL126, in which there was a 22-fold increase in proline levels upon osmotic stress. In this strain in 0.65 M NaCl, proline constituted 61% of the free ninhydrin positive organic molecules (data not shown). This buildup of proline might be accomplished by increased synthesis, or decreased degradation, or an enhancement of the ability of cells to retain high levels of proline. Experiments distinguishing between these alternatives are in progress. The third point about Table 4 is that high intracellular proline levels are at least qualitatively correlated with enhanced osmotolerance: in the presence of 0.65 M NaCl, the proline level in the markedly osmotolerant strain TL126 was 8- to 35-fold greater than the proline levels in the other strains which exhibited slight or no enhanced osmotolerance.

Discussion

The main finding presented in this publication is that a class of spontaneous mutations which enhance the osmotolerance of *Salmonella typhimurium* can be easily generated. These mutations were obtained as alterations resulting in proline over-production, suggesting that the enhanced osmotolerance might be a consequence of the high intracellular levels of proline. The mutations map in the vicinity of the *proB*$^+$*A*$^+$ genes, and upon transfer to other *Salmonella* strains, they confer the phenotype found with the original hosts.

The osmotolerant mutants were generated by an indirect route. A preliminary attempt to obtain strains with enhanced osmotolerance by direct selection was unsuccessful, but it is an interesting question whether the phenotype could be conferred by other kinds of mutations. It is curious that of the 107 mutants (plus an additional one obtained from Dr. John Roth) with identical resistance to L-azetidine-2-carboxylate caused by a mutation linked to *proBA*, only one, TL126, proved to have acquired markedly enhanced osmotolerance. At present, we do not know whether the alteration in this strain is of a more extreme form or of a different kind than those found in the others. Proline over-production might be due to desensitization of the first enzyme of the proline biosynthetic pathway to feedback inhibition by proline (Condamine 1971) or it might be the result of elevated synthesis of one or more of the proline biosynthetic enzymes.

Our data indicate that increased intracellular proline levels result in enhanced osmotolerance. The proline level, in the presence of 0.65 M NaCl, in the markedly osmotolerant strain TL126 was over 400-fold greater than in the control strain TL128; the proline levels under the same condition in the slightly osmotolerant strains TL123 and TL124 were at least 50- and 39-fold greater, respectively, than in strain TL128 (Table 4).

Our most interesting result is that the proline levels in the proline over-producing strains seem to be regulated so that they increase (up to 22-fold in TL126) in response to 0.65 M NaCl. One explanation for this phenomenon could be that the mutations have unmasked a cryptic regulatory mechanism modulating the rate of proline biosynthesis in response to the external osmotic stress. Another explanation could be that the intracellular proline levels in the mutants are regulated by the activity of the proline transport system. Working with *E. coli*, Britten and McClure (1962) found that the uptake and accumulation of proline from the exterior was stimulated in direct proportion to the osmotic strength of the growth medium. It is possible that proline, synthesized at excess in our mutants, is excreted and then taken up again, so its internal concentration is regulated by the transport systems, in response to the external osmolarity. The enhanced uptake of proline might result in increased osmotolerance either because it may serve as an additional osmotic balancer (see below), or because its transport is coupled to Na^+ uptake (and also K^+ to a lesser extent) (Kayama and Kawasaki 1976), which might function to neutralize the negatively charged amino acids used as osmotic balancers (see below).

In a wide variety of organisms, the internal concentrations of a number of inorganic ions and low molecular weight compounds were found to increase in response to osmotic stress (Flowers et al. 1977; Hellebust 1976). An explanation offered for this phenomenon is that the increase in the total concentration (or more precisely the activity) of these solutes serves to maintain the osmolarity of the interior to equal or exceed the osmolarity of the exterior and thus forestall the dehydration of the cytoplasm (Measures 1975; Flowers et al 1977; Hellebust 1976). We found that the levels of glutamine and glutamic acid increase in *Salmonella typhimurium* in response to osmotic stress. Thus these two compounds could comprise some of the osmoregulatory substances used by this organism. Proline, which does not normally play a role in osmoregulation, may apparently be made to do so in the osmotolerant mutants. As suggested above, the modulation of the proline levels might be mediated by the transport system(s), which could be a reflection of the fact that even wild type *Salmonella typhimurium* (and other bacteria, cf. Measures 1975) might use proline as an osmoregulatory substance if it is present in the growth medium.

Although there does not seem to be one universal mechanism of osmoregulation, and in various organisms inorganic ions (Christian and Waltho 1961; Epstein and Schultz 1965), amino acids (Measures 1975; Flowers et al. 1977; Hellebust 1976; Tempest et al. 1970; Brown and Stanley 1972; Makemson and Hastings 1979), polyols (Flowers et al. 1977; Hellebust et al. 1976), polyamines (Munro et al. 1972), and quaternary amines (Wyn Jones 1980) were suggested to act as osmotic balancers, nevertheless proline is a prominent substance to be accumulated in response to osmotic stress in a wide variety of organisms, ranging from bacteria (Measures 1975; Tempest et al. 1970; Brown and Stanley 1972) to algae (Schobert 1977a; Brown and Hellebust 1978), and higher plants (Flowers et al. 1977; Stewart and Lee 1974; Cavalieri and Huang 1979; Tully et al. 1979). Schobert (1977b) put forward a hypothesis for the advantages of proline as an osmoregulatory substance: that it has special

interactions with proteins which enhances their stability and solubility in solutions of low water activity (cf. also Schobert and Tschesche 1978). Should this suggestion be corroborated, it would be of great importance because it would mean that the accumulation of proline at high levels would be sufficient to cause enhanced osmotolerance. In that case, the selection scheme we have employed with *Salmonella typhimurium* might be adapted to generate osmotolerant derivatives of other bacterial species, cultured plant cells, or possibly whole plants.

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