# Molecular Biology of Osmoregulation

D. Le Rudulier, A. R. Strom, A. M. Dandekar
L. T. Smith, R. C. Valentine

*Summary.* The drought of 1983 resulted in some 10 billion dollars in agricultural losses and has focused attention on the vulnerability of our major crops to this devastating form of environmental stress. This article is concerned with the molecular biology of a new class of genes, called *osm* (osmotic tolerance) genes, that protect bacteria like *Escherichia coli* against osmotic stress and may work in a similar manner in plants and animals. *Osm* genes govern the production of a class of molecules, such as betaine and proline, that protect the cell and its constituents against dehydration. These osmoprotectant molecules have been known for many years to accumulate in plants but have only recently been shown to have potent antistress activity for bacteria.

Cellular adaption to osmotic stress is a cardinal biological process that protects organisms against the lethal effects of dehydration. Osmoregulation is of great significance in agriculture, since water is the major limiting factor in crop productivity. The severe drought in the United States in 1983, which resulted in crop losses estimated at more than $10 billion (48 percent of the total corn crop was lost and 33 percent of the soybeans), called public attention to the vulnerability of our major crops to drought. However, even in an average year the usual series of mini-droughts during the growing season may represent one of the most significant losses in agricultural productivity. Boyer (*1*) has pointed out that from 1939 to 1978 over 40 percent of the total insurance indemnities for crop losses involved drought. Salinity is another form of water-related stress responsible for major crop losses worldwide, especially in semiarid and irrigated agriculture (*2*). In major agricultural states like California and Texas, salinity is regarded as the most pervasive problem of irrigated agricultural lands.

Plants have evolved a variety of mechanisms for adapting to osmotic stress; in this article we focus on cellular adaptation to osmotic stress (osmoregulation). Many investigators (*3–9*) have hypothesized that a class of small organic molecules (glycine betaine, proline betaine, proline, and others) found in scores of higher plants behave as osmoprotectants (Fig. 1). They propose that the molecules accumulate in plant cells during osmotic stress and prevent damage from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment. Evidence that correlates the increase in glycine betaine or proline betaine accumulation in higher plants with the increase in NaCl concentration is now available for a number of evolutionarily diverse plants (*4, 7, 9*).

The metabolism of the putative osmoprotective molecules in plants has been studied in some detail (*10–12*). It is recognized that accumulation of osmoprotectants against stress would require fixed carbon and nitrogen, thus utilizing valuable resources of the plant. However, several researchers (*4, 7, 9*) have proposed that this cost may be minimized by localization of osmoprotectants in key regions of the plant cell.

How does the cell sense osmotic change? Bacterial studies have recently shed light on this aspect of osmoregulation. For example, the molecular basis of osmosensory systems is being actively researched in several laboratories, with the porin and potassium uptake systems of *Escherichia coli* receiving the most attention. Porins, a class of outer membrane proteins in *E. coli*, form passive diffusion pores that allow hydrophilic compounds of low molecular weight to cross the outer membrane (*13*). The osmolarity of the medium strikingly affects the proportionate amount of two porins, OmpC and OmpF, such that the *ompC* gene is preferentially expressed in cells grown in a medium of high osmolarity while the *ompF* gene is expressed in cells grown in a medium of low osmolarity. Hall and Silhavy (*14*) proposed that an osmosensory protein embedded in the envelope functions to trigger a regulatory cascade involving a second, cytoplasmic regulatory protein that causes selective expression of the porin genes.

Laimins *et al.* (*15*) showed that potassium transport plays a vital role in osmoregulation in *E. coli*. These investigators suggested that a membrane-bound osmosensing protein regulates potassium transport genes as a function of osmotic strength. They tested this model by genetically fusing the promotor region of an osmotically sensitive potassium uptake gene cluster with the structural region for β-galactosidase and observing that β-galactosidase production was now regulated by the osmotic strength of the environment. Kennedy (*16*) proposed that an "osmotic sensor" protein in the inner membrane of *E. coli* plays a crucial role in modulating the biosynthesis of a class of oligosaccharides whose production is geared to the osmotic strength of the environment. He further proposed that these unique oligosaccharides may act as polymeric osmoprotectants and play a key role in osmoregulation. It will be of considerable interest to determine whether any of the newly discovered osmotically modulated systems discussed below share a common osmosensor with these systems.

In this article we summarize recent advances in understanding both the mechanism of cellular adaptation to osmotic stress and the role of osmoprotective compounds and their genes in uptake and synthesis of osmoprotective molecules. Once again, bacteria have played a key role in these studies. The most interesting finding is that several of the molecules that accumulate during osmotic stress in plants also behave as potent osmoprotectants for bacteria (*17, 18*). Studies of the biosynthesis, uptake, and degradation of these molecules strongly support their biological role as osmoprotectants in bacteria. A gene governing overproduction of one of the mol-

D. Le Rudulier is a professor in the Laboratoire de Physiologie Végétale, Université de Rennes I, Campus de Beaulieu, 35042 Rennes Cedex, France. A. R. Strom is a professor at the Institute of Fisheries, University of Tromso, P.O. Box 3083 Guleng, N-9001 Tromso, Norway. A. M. Dandekar, L. T. Smith, and R. C. Valentine are research molecular biologist, research biochemist, and professor, respectively, in the Plant Growth Laboratory and Department of Agronomy and Range Science, University of California, Davis 95616.

ecules, proline, has been cloned and confers osmotic tolerance in several species of bacteria (*17*). In addition, the process of biological nitrogen fixation in the free-living bacterium *Klebsiella* has been shown to be markedly protected from osmotic stress by proline (*19*), glycine betaine (*20*), and proline betaine (*21*).

In addition to functioning as osmotic balancing agents, there is increasing evidence that osmoprotectants may interact with crucial macromolecules of the cell to help modulate their biological activity. For example, Yancey *et al.* (*3*) have proposed that osmoprotectants may act both as nonperturbing or compatible solutes and as stabilizers of protein structure and function. To support this idea, these workers cited a variety of organisms and enzymes that span a wide evolutionary spectrum. Convincing arguments for the selective advantage of organic osmoprotectants over commonly available salts are presented with an evolutionary perspective.

The following sections address the crucial role played by osmoprotectant molecules in many different organisms. Those molecules possessing potent biological activity when supplied exogenously to stressed bacteria are emphasized. Undoubtedly this list will be expanded to include other "osmolytes" known to accumulate in stressed cells (*3*, *9*).

## Osmoprotectants

Many organisms have learned to use a simple rule of chemistry to live in a world deficient in available water. They have evolved sophisticated mechanisms for balancing their osmotic strength with that of their surroundings. In other words, they are able to avoid dehydration by taking up or synthesizing molecules that act as osmotic balancing agents. Theoretically, a vast number of compounds could serve this function. However, an organism may use only a few compounds to fill this need.

As illustrated in Fig. 1, *E. coli* grows under completely inhibitory levels of osmotic strength when supplemented with osmoprotectants such as glycine betaine (Fig. 2). The petri dishes shown were all inoculated with an equal number of bacteria and all contained NaCl at completely inhibitory osmotic strength (0.8*M*). The compounds added to the upper set of dishes did not support growth, while the dishes supplemented with some of the more potent osmoprotectants, such as glycine betaine and its precursor choline, protected the bacteria against osmotic stress. The purpose of this experiment was to test the influence of methyl groups on the potency of osmoprotection; for example, glycine betaine is a trimethylated derivative of the amino acid glycine, with the three methyl groups attached to the nitrogen. Choline has a similar structure, except that the carboxyl function of glycine is replaced by an alcohol function. Choline shows activity while dimethylglycine shows only traces of activity. Neither glycine nor its monomethyl derivative sarcosine shows activity. γ-Aminobutyric acid was another negative control showing no activity. Of approximately 150 different metabolites tested so far, only the betaine series proved potent in promoting growth under strongly inhibitory levels of osmotic strength. The most active molecules include glycine betaine, choline, and proline betaine; free proline is also active but under lower levels of stress. Trimethyl-γ-aminobutyric acid is also active (Fig. 2).

How do osmoprotectants work? The simplest explanation is that they allow the cell to balance the osmotic strength of its cytoplasm with that of its surroundings to prevent a net loss of water. However, recent experiments suggest that osmoprotectants may have other functions as well. For example, osmoprotectants may influence protein structure and stability. During severe stress in bacteria, cellular constituents may literally be bathed in osmoprotectants that reach concentrations above 1*M*. In the case of proline, Schobert and Tschesche (*22*) found that proline solutions interact with proteins, increasing the solubility of sparingly soluble proteins and reducing the precipitation of soluble proteins by ethanol and $(NH_4)_2SO_4$. They postulated that the water-binding capacity of the proline-protein is increased. Studies of mutationally altered enzymes whose ac-

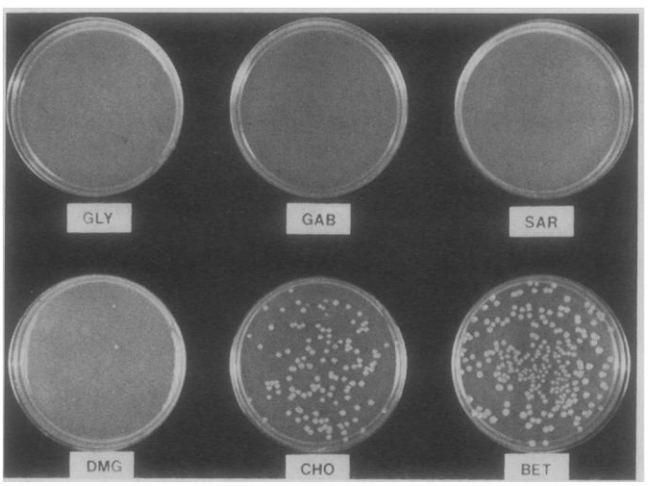



Fig. 1. Betaines as osmoprotectants for *E. coli*. Seeds, plants living in seawater, and plant tissues subjected to drought or salinity often contain relatively high levels of osmoprotectants. Molecules are considered to be osmoprotectants only when they display biological activity in permitting cells, such as the bacteria shown here, to grow at normally inhibitory levels of osmotic strength. The petri dishes contain a chemically defined growth medium supplemented with inhibitory levels of osmotic strength (0.8*M* NaCl; almost double the osmotic strength of seawater). Glycine betaine (*BET*) or its derivatives choline (*CHO*), glycine (*GLY*), sarcosine (*SAR*), or dimethylglycine (*DMG*) were added at a concentration of 0.001*M*. The inoculant was *E. coli* K10.

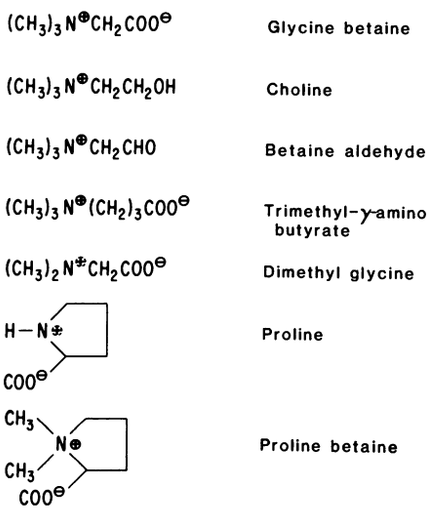



Fig. 2. Structures of osmoprotectants of *E. coli* and other enteric bacteria. One of the striking chemical features of molecules like glycine betaine and proline is their extreme solubility in water (about 14 kilograms per kilogram of water). In addition, they possess other unique chemical activities suited for their role as osmoprotectants (*3*). On a global scale, plant life (including marine forms) may be the major source of betaines as osmoprotectants in the biosphere.

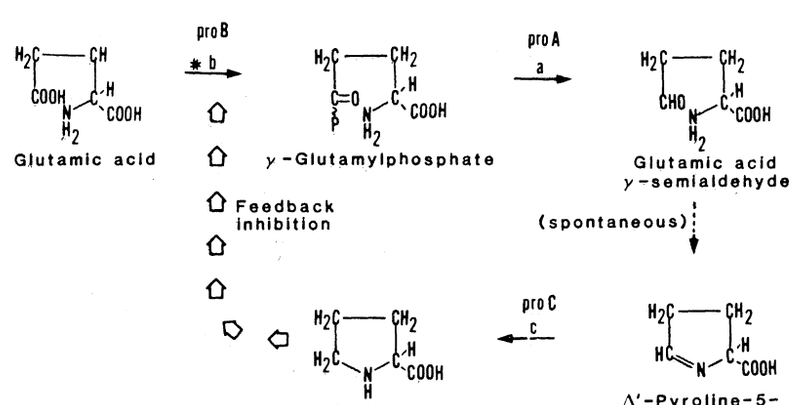
tivities are stabilized or destabilized in vivo by perturbation of osmotic strength also support the important role played by osmoprotectants in protein structure and function (*18, 23, 24*). Clearly, the effect of osmoprotectants on the physicochemical properties of proteins and other cellular constituents is an important area for future research.

### *Osm* Genes

*Escherichia coli* is a convenient organism for studying osmotic tolerance (*osm*) genes because of the advanced state of its genetics and because it has evolved a sophisticated system for protecting against osmotic stress. Indeed, *E. coli* behaves like a miniature osmometer, sensing and responding to changes in the osmotic strength of its environment.

The strategy of investigators in this field has been to construct and characterize mutants in all functions essential for osmotic tolerance and osmoregulation, including osmosensory or "trigger" steps as well as metabolic stages. Our approach, described below, has been to focus on *E. coli* and the contribution of two specific compounds, glycine betaine and proline, that confer osmotic tolerance. Two different classes of mutants will be discussed, proline-overproducing mutants that simultaneously show osmotic tolerance and mutants blocked in the synthesis of glycine betaine from choline.

The properties of a proline-overproducing mutation that confers osmotic tolerance are summarized in Fig. 3. To our knowledge, this is the first example of a mutation (gene) conferring osmotic tolerance (*25*). The proline-overproducing mutant was selected by using the proline analog L-azetidine-2-carboxylate (*25*). Proline overproducers, which can dilute the analog, survive the selection.



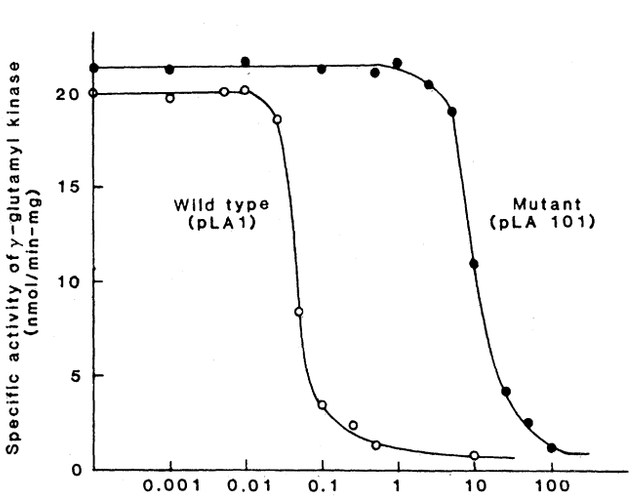




Fig. 3. A rare, proline-overproducing mutation conferring osmotic tolerance (*25*). The three enzymes in this pathway are γ-glutamyl kinase (*b*), γ-glutamylphosphate reductase (*a*), and Δ'-pyrroline-5-carboxylate reductase (*c*), which are coded for by the *pro*B, *pro*A, and *pro*C genes, respectively. The asterisk indicates the reaction that is modified in the mutant, where γ-glutamyl kinase is less sensitive to feedback inhibition. The lower curve shows that the mutant enzyme is markedly less sensitive to inhibition by proline.

The DNA fragment which allows the overproduction (that is, which contains the *osm* genes) has been cloned and contains the *pro*AB genes but not *pro*C (*17*). Using partially purified enzyme or crude extracts from bacteria with the cloned genes and, therefore, with amplified levels of enzyme, we have found that the γ-glutamyl kinase from the mutant is about 100 times less sensitive to the presence of proline than is the wild-type enzyme (bottom curve in Fig. 3). We propose that this accounts for proline overproduction and leads to osmotic tolerance in the mutant strain. This mutation, which results in a more than 125-fold increase in the concentration of intracellular proline, is rare; most proline-overproducing strains do not show increased osmotic tolerance.

To provide a framework for discussing the area of glycine betaine (choline), glycine betaine metabolism in *E. coli* (Fig. 4) will be summarized. Imagine that *E. coli* are subjected to osmotic stress in a medium containing glycine betaine. To be effective, glycine betaine must be supplied in the medium or one of its precursors (choline or betaine aldehyde) must be supplied (choline is effective only if the medium is aerated). Stimulation of growth occurs with exogenous concentrations of glycine betaine in the range $10^{-5}$ to $5 \times 10^{-4} M$. Glycine betaine accumulates intracellularly in very high concentrations as a function of external osmotic strength (Fig. 5). For example, at very high external osmotic strengths (for example, 1*M* NaCl), *E. coli* concentrates glycine betaine in the cell to $10^5$ times the level found in the medium.

The nature of this osmotically modulated uptake system is of considerable interest. Development of a rapid and sensitive uptake assay with radioactive glycine betaine has led to a better understanding of the system. Recent results in *E. coli* (*26*) show that only cells grown under osmotic stress accumulate significant levels of glycine betaine (Fig. 5), suggesting that osmotic stress alone somehow activates or causes expression of this uptake system. (It is premature to use the word induction, since the cellular level at which activation takes place is not yet known.) A variety of salts (NaCl, KCl, and others) as well as nonelectrolytes (such as sugars) trigger the uptake of glycine betaine, suggesting a generalized osmosensor. Structure-function studies with structural analogs of glycine betaine reveal that the uptake system is specific for glycine betaine.

The glycine betaine uptake system in

*E. coli* can be used to study regulation of membrane functions associated with osmotic tolerance. The system may be part of a network of osmotically regulated uptake systems for osmoprotectants (*27, 28*).

Choline and betaine aldehyde are effective in protecting *E. coli* against osmotic stress (Fig. 4). However, these molecules are not osmoprotective per se, since tracer studies reveal that they are rapidly converted to and stored as glycine betaine in the cytoplasm (*29*). Mutants blocked in this pathway are not protected against osmotic stress by choline. Conversion of choline to betaine aldehyde is catalyzed by a membrane-associated, $O_2$-dependent enzyme, whereas a soluble dehydrogenase dependent on nicotinamide adenine dinucleotide phosphate is responsible for oxidation of the aldehyde to the end product, glycine betaine (*29*). This pathway is osmotically regulated. That is, both enzyme activities appear simultaneously when the osmotic pressure of a medium containing choline is increased by addition of an osmolyte (salts or sugars). Pathways for synthesis of glycine betaine from choline have been described in several species of bacteria, but this oxidation was not previously related to osmotic tolerance.

Mutants have been selected in the *bet/osm* pathway that phenotypically are not protected against osmotic stress by choline but display osmotic tolerance when supplied with glycine betaine in the growth medium. Biochemical analysis reveals that the mutants are blocked in one or more of the steps of choline metabolism (uptake, oxidation, or dehydrogenation).

Three different genetic procedures were used to map these mutations on the genetic linkage map of *E. coli* (*30*): P1 transductional analysis, deletion analysis, and F-prime complementation analysis. For the F-prime analysis, a mutant of *E. coli* was chosen that has a natural deletion of the *lac* genes (lactose utilization genes) and their flanking DNA sequences. Physiological and biochemical analyses revealed that this strain also lacks choline uptake enzymes and enzymes for converting choline to glycine betaine, but retains the ability to grow at high osmotic strength when supplied with glycine betaine. The introduction of a segment of DNA mirroring the missing region on a F-prime *lac-pro* plasmid restored all these missing activities to their normal state. These and other experiments led to the conclusion that the *bet/osm* genes crucial for osmoprotection with choline are located a short distance proximal to the *lac* operon on the *E. coli* linkage map (Fig. 6). These findings have set the stage for molecular cloning and fine-structure genetic analysis of the *bet/osm* region.

To our knowledge the *bet/osm* genes are the first set of naturally occurring genes for osmotic tolerance that have been identified in any organism and should provide valuable insight on how osmotically regulated systems work. In addition, they may serve as a source of regulatory elements and genes for use in genetic engineering.

## Osmoregulation in Plants

Do plants and bacteria share a common mechanism of osmoprotection? Le Rudulier and co-workers (*31, 32*) recently described preliminary experiments in which betaines protected both the free-living root nodule bacteria of alfalfa as well as nodulated seedlings. This experiment was done in two parts. In the first part, betaines protected the symbiotic bacterium of alfalfa, *Rhizobium meliloti*, against stress, leading to a picture of osmoprotection in this organism which parallels that in *E. coli*. One difference

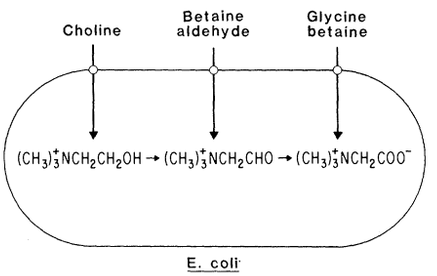



Fig. 4. Choline–glycine betaine metabolism in *E. coli*: an example of an osmotically modulated pathway. Choline and glycine betaine are metabolically linked, with the overall system, including uptake and metabolism, being modulated by the osmotic strength of the environment.

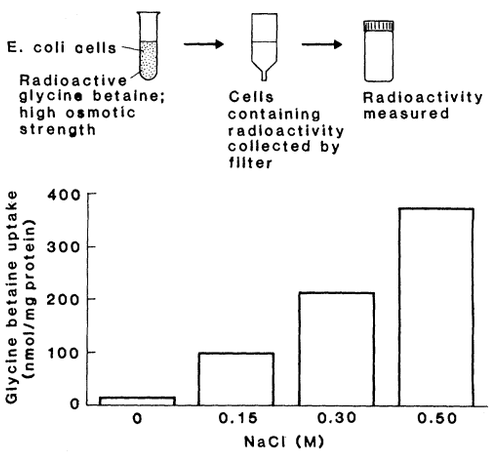



Fig. 5. Osmotically regulated uptake of glycine betaine in *E. coli*. (a) A rapid and sensitive uptake assay based on the use of radioactive glycine betaine (*26*). The steps include incubation of 1 ml of previously stressed cells (a prerequisite for appearance of this activity) with [$^{14}$C]glycine betaine (64,000 disintegrations per minute per assay) and collection of the cells with a membrane filter. The filter is washed with an isotonic solution, since intracellular glycine betaine is lost if the wash solution is of lower osmotic strength than the cell. (b) A bar graph showing that the level of glycine betaine maintained in the cell is a function of osmotic strength of the medium. The assay method described above was used to follow the kinetics of [$^{14}$C]glycine betaine (0.25 m*M*) uptake in order to determine the steady-state level of glycine betaine in *E. coli* K10 (*26*).

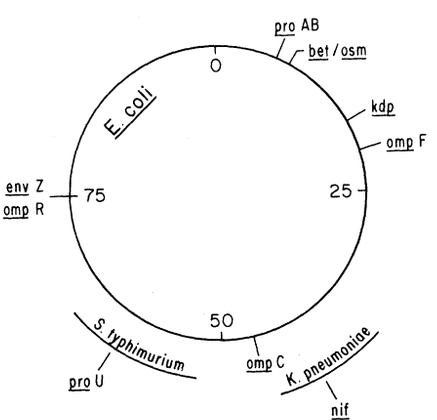



Fig. 6. Molecular biology of osmoregulation genes in *E. coli*. The figure summarizes the various genes of *E. coli* and its relatives implicated in adaptation to osmotic stress. The approximate locations of these genes on the genetic linkage map of *E. coli* are shown. The genes, in clockwise order around the chromosome, include *pro*AB (proline overproduction); *bet/osm* (choline–glycine betaine); *kdp* (potassium uptake); *omp*F, *omp*C, *omp*R, and *env*Z (porin synthesis and control); *pro*U (proline uptake); and *nif* (nitrogen fixation). Osmotic stress inhibits synthesis of nitrogenase polypeptides, making this system another example of a set of genes switched off by stress.

worth mentioning is that, in the absence of osmotic stress, *R. meliloti* but not *E. coli* actively catabolizes glycine betaine, salvaging carbon and nitrogen. Interestingly, this catabolic degradation system is "repressed" by increasing the osmotic strength of the medium, preventing a futile cycle of uptake and degradation and ensuring that glycine betaine is preserved to function as an osmoprotectant.

The bacterial experiment served as a bridge to the whole-plant study in which glycine betaine as well as proline betaine supplied to the roots of nodulated alfalfa seedlings enhanced symbiotic nitrogen fixation. For example, symbiotic $N_2$ fixation by control plants exposed to 0.2*M* NaCl was almost completely blocked for $N_2$ fixation activity. On the other hand, stressed plants treated with 10 m*M* betaines recovered up to 30 percent of maximum levels observed with the untreated controls. It is interesting to speculate that betaines are behaving as osmoprotectants at the whole-plant level, perhaps protecting the $N_2$-fixing apparatus in the nodule tissue against stress.

## Conclusion

Cellular adaptation to osmotic stress is a fertile area for basic research, with possible future applications in agriculture, medicine, and industry (*33, 34*). The concept of osmosensory proteins linking environmental changes in osmotic strength to dynamic changes in membrane, biochemical, and genetic activities of the cell should generate further investigations by molecular biologists and biochemists. The discovery of osmoprotective molecules and new classes of *osm* genes may lead to genetic enhancement of drought and salinity tolerance in crop plants. To achieve this challenging goal the emerging technology must be closely integrated with established procedures of plant improvement.

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35. This article was prepared with the support of NSF grant PFR 77-07301. D.L.R. was supported by a grant from Centre National de la Recherche Scientifique and A.S. by a grant from the Norwegian Research Council of Fisheries. We thank B. Perroud, T. Bernard, J.-A. Pocard, L. Bouillard, G. Goas, B. Landfald, P. Falkenberg, T. Bjørnsen, O. B. Styrvold, M. Jakowec, and M. Eshoo for theses and preprints.


**RESEARCH ARTICLE**

# Structure and Expression of a Complementary DNA for the Nuclear Coded Precursor of Human Mitochondrial Ornithine Transcarbamylase


Arthur L. Horwich, Wayne A. Fenton, Kenneth R. Williams
Frantisek Kalousek, Jan P. Kraus, Russell F. Doolittle
William Konigsberg, Leon E. Rosenberg


Systems of compartmentation in eukaryotic cells direct specific polypeptides synthesized in the cytoplasm to destinations that include the extracellular space, cellular membranes, lysosomes, and the mitochondria. The first three of these systems share a common feature; polypeptides are synthesized on membrane-bound polyribosomes and are cotranslationally inserted into the cisternae of the endoplasmic reticulum (*1*). The system responsible for the compartmentation of nuclear-coded mitochondrial proteins appears to be fundamentally different; these polypeptides are synthesized on free polyribosomes and are subsequently released into the cytoplasm for posttranslational import by the mitochondria (*2*).

This import process is a major determinant of mitochondrial biogenesis. Fewer than 20 mitochondrial polypeptides are encoded by mitochondrial DNA and synthesized on mitochondrial ribosomes (*3*). The remainder (more than 200) are encoded in the nucleus and synthesized in the cytoplasm. Most nuclear-coded polypeptides destined for mitochondria are synthesized in the cytoplasm as larger precursors, containing amino-terminal leader sequences not present in their mature mitochondrial counterparts. Import of these precursors involves specific binding by receptor molecules present in the outer mitochondrial membrane, translocation through one or both mitochondrial membranes


A. L. Horwich, W. A. Fenton, F. Kalousek, J. P. Kraus, and L. E. Rosenberg are in the Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510. K. R. Williams and W. Konigsberg are in the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine. R. F. Doolittle is in the Department of Chemistry, University of California, San Diego, La Jolla, California 92093.