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Dimethylthetin Can Substitute for Glycine Betaine as an Osmoprotectant Molecule for *Escherichia coli*

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Glycine betaine is believed to be the most active naturally occurring osmoprotectant molecule for *Escherichia coli* and other bacteria. It is a dipolar ion possessing a quaternary ammonimum group and a carboxylic acid group. To examine the molecular requirements for osmoprotective activity, dimethylthetin was compared with glycine betaine. Dimethylthetin is identical to glycine betaine except for substitution of dimethyl sulfonium for the quaternary nitrogen group. Dimethylthetin was found to be about equally as effective as glycine betaine in permitting *E. coli* to grow in hypertonic NaCl, and both compounds were recovered almost completely from bacterial cells grown in the presence of hypertonic NaCl. 3-Dimethylsulfonioproprionate, an analog of dimethylthetin observed in marine algae, and 3-methylsulfonio-2-methylproprionate were found to be less active. Dimethylthetin may prove useful as a molecular probe to study betaine metabolism and as a model for the development of antibacterial agents.

Escherichia coli is protected from external osmotic forces by a series of adaptive mechanisms controlled by osmotic-tolerance genes (9). Osmosensitive proteins, located in the outer membrane, trigger regulatory responses to osmotic stress. These responses include alterations in porin proteins (10, 15), potassium transport (7, 14), and uptake and synthesis of osmoprotective molecules. The major osmoprotective substances are betaine, proline, and glutamate. They are termed compatible solutes because they accumulate intracellularly in the presence of high concentrations of extracellular solutes and protect the cell from dehydration. Glycine betaine is the most important of these compounds, apparently because, in part, of its dipolar characteristics and high solubility in water. Glycine betaine has been shown to be synthesized by halophilic eubacteria in direct response to the external osmotic pressure (6).

Compatible solutes appear to be osmoprotective for vascular plants and animals (16). Several species of marine algae have been reported to contain glycine betaine, stachydrine (proline betaine), *trans*-4-hydrostachydrine, and 3-dimethylsulfonioproprionate (dimethyl-beta-propriothetin) (2). The extremely eurohaline mollusc *Elysia chloritica* survives in salinities ranging from 24 to 2,422 mosM and utilizes proline betaine for osmoregulation. The crab *Limulus polyphemus* uses glycine betaine to sustain cell volume in hypertonic salt water (13). Glycine betaine and proline betaine have been recovered from human urine (3) and appear to play an important role in osmotic protection of the mammalian renal papilla (1, 4).

Dimethylthetin (also known as dimethylacetothetin or sulfobetaine) differs from glycine betaine in the substitution of a positively charged dimethyl sulfur moiety for the quaternary nitrogen group (Fig. 1). It can substitute for glycine betaine and choline as an efficient methyl donor in mammals (11). It is chemically related to naturally occurring 3-dimethylsulfonioproprionate found in marine algae. We therefore sought to determine whether these sulfur-containing analogs could substitute for glycine betaine and proline betaine as osmoprotective agents for *E. coli*.

Glycine betaine was obtained from Sigma Chemical Co., St. Louis, Mo. Proline betaine was synthesized by the method of Cornforth and Henry (5). Dimethylthetin was obtained from Chem Services Co., West Chester, Pa. 3-Dimethylsulfonioproprionate and 3-dimethylsulfonio-2-methylproprionate were synthesized by allowing 5 ml (68 mmol) of methyl disulfide to react with 2 ml (29 mmol) of acrylic acid or methacrylic acid in 30 ml of methylene chloride. Hydrogen chloride gas was bubbled through the solution with stirring for 20 min. The resulting solid was isolated by filtration and then recrystallized from methanol-ether. The melting point was 125 and 126°C, respectively, for the two compounds. (8). *E. coli* 31, which was used in previous experiments (3), was grown in medium containing 2.9 g of glucose, 7.0 g of K_2PO_4, 3.0 g of $KHPO_4$, 0.5 g of trisodium citrate, 0.5 g of $MgSO_4$, and 1.0 g of NH_4SO_4 per liter (3). The osmolality as measured by freezing point depression was 178 mosM/kg. NaCl was added at various concentrations, and the pH was adjusted to 7.0. The bacterial inoculum consisted of 0.01 ml of a 1:100 dilution of an overnight culture to 1 ml of the mixture to be tested. Cultures were incubated at 37°C. Bacterial growth in experiments to determine maximal salt tolerance was determined by visual examination for turbidity after incubation for 24 and 48 h. Bacterial growth curves were obtained by recording light scatter (optical density) at 400 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

The uptake of glycine betaine and dimethylthetin by *E. coli* was determined by growing the organism in minimal medium containing 0.9 M NaCl together with the osmoprotectant compounds. The compounds were added to separate flasks at a final concentration of 10^{-4} M. The cultures were grown at 37°C for 48 h, and the cells were harvested by centrifugation. The pellets were suspended in 3 ml of water and divided into three aliquots, which were then boiled for 10 min and sonicated for another 10 min. After centrifugation, the supernatants were lyophilized to dryness and extracted with methanol. The methanol fraction was then removed and dried under a stream of nitrogen. The residue was dissolved in water, and a sample was applied to a C3

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$(CH_3)_2\text{-}\overset{+}{S}\text{-}CH_2\text{-}COO^-$ Dimethylacetothetin

$(CH_3)_3\text{-}\overset{+}{N}\text{-}CH_2\text{-}COO^-$ Glycine Betaine

FIG. 1. Chemical structures of glycine betaine and dimethylthetin.

reverse-phase high-pressure liquid chromatography column (10 by 250 mm; Alltech Associates, Inc., State College, Pa.) in combination with a Hewlett-Packard 1080B instrument. The mobile phase was pure water. The elution profile was monitored for UV absorbance at 205 nm. Under these conditions, dimethylthetin emerged several minutes before glycine betaine as a distinct peak. Standard curves (r = 0.999) were prepared for each compound. To determine uptake from the medium, samples were obtained before the organism was added and from the supernatant at 48 h of growth. These fluids were divided into three aliquots, lyophilized to dryness, and extracted with methanol. Glycine betaine and dimethylthetin were measured as described above.

Dimethylthetin and glycine betaine supported bacterial growth over a similar concentration range and similar sodium chloride stress (Table 1). 3-Dimethylsulfonioproprionate and 3-methylsulfonio-2-methylproprionate were less active than glycine betaine. The addition of the 2-methyl group had no apparent effect on osmoprotective activity. Growth of the test strain of *E. coli* in the presence of 10^{-4} M concentrations of glycine betaine, proline betaine, and dimethylthetin at various concentrations of NaCl, as compared with that of control cultures, is shown in Fig. 2. The osmoprotective activities of the compounds were virtually identical except for a slightly greater yield of the test organism at 1.0 M NaCl in the presence of dimethylthetin.

Recovery of the compounds from minimal medium before inoculation of the *E. coli* strain was 91.3 and 83.6% for glycine betaine and dimethylthetin, respectively. At 48 h of incubation, 101.2% of glycine betaine and 92.4% of dimeth-

TABLE 1. Growth of *E. coli* in minimal medium in the presence of NaCl

Osmoprotectant[a]	Highest NaCl concn (M) in which growth occurred at the following osmoprotectant concn (M):					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	None[b]
Glycine betaine	1.0	1.0	1.0	0.9	0.7	0.7
Dimethylthetin	1.0	1.0	1.0	0.9	0.7	0.7
2-DMSP	0.8	0.8	0.8	0.7	0.7	0.7
2-DMS-2M-P	0.8	0.8	0.8	0.7	0.7	0.7

[a] 2-DMSP, 3-Dimethylsulfonioproprionate; 2-DMS-2M-P, 3-methylsulfonio-2-methylproprionate.

[b] None, Minimal medium alone.

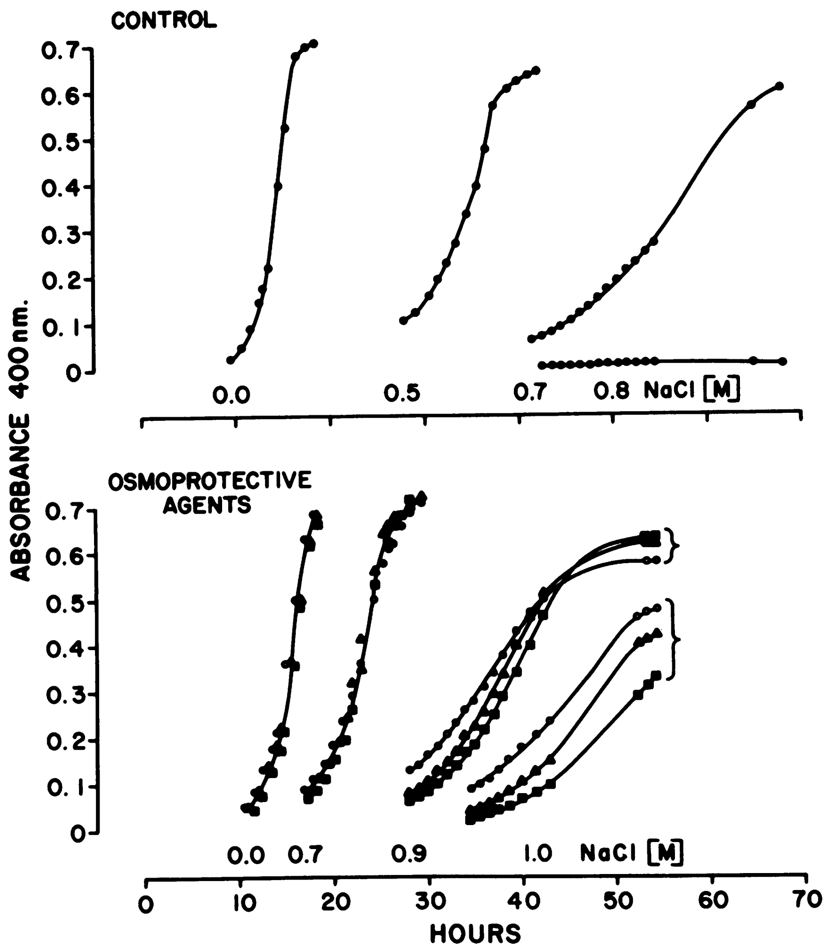

FIG. 2. Growth of *E. coli* in minimal medium at 37°C in the presence of various concentrations of NcCl alone (control) or with 10^{-4} M glycine betaine (▲), proline betaine (■), or dimethylthetin (○), as measured by optical density.

ylthetin were recovered from the bacterial cell pellet. Neither compound was detectable in the supernatent medium (the sensitivity of detection was less than 10^{-7} M).

It is apparent from these experiments that *E. coli* does not seem to distinguish between betaines containing a sulfur or a nitrogen group. The compounds supported growth in hypertonic NaCl about equally well, and both were removed from the medium and recovered from the bacterial cells. As previously noted for the nitrogen compounds (12), the length of the carbon chain influences the biological activity of the sulfur-containing compounds. Dimethylthetin may serve as a useful probe to study intracellular synthesis and storage of betaines as compared with uptake and excretion into the medium. It is also possible that analogs of these compounds which can block uptake of betaines may be synthesized. We have found in preliminary studies that staphylococci and enterococci, as well as gram-negative enteric bacteria, accumulate glycine betaine under osmotic stress. It may be possible to use the avid betaine uptake mechanism to deliver lethal antibacterial agents intracellularly. This opens the possibility of developing antibacterial agents which are active in hypertonic urine.

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