



Research review paper

Ectoines in cell stress protection: Uses and biotechnological production

José M. Pastor^{a,1}, Manuel Salvador^{b,1,2}, Montserrat Argandoña^{b,2}, Vicente Bernal^a, Mercedes Reina-Bueno^{b,2}, Laszlo N. Csonka^c, José L. Iborra^a, Carmen Vargas^{b,2}, Joaquín J. Nieto^{b,2}, Manuel Cánovas^{a,*}

^a Department of Biochemistry and Molecular Biology B and Immunology, University of Murcia, Campus de Espinardo, 30100 Murcia, P.O. Box 4021, Spain

^b Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville, Spain

^c Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

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ABSTRACT

Microorganisms produce and accumulate compatible solutes aiming at protecting themselves from environmental stresses. Among them, the wide spread in nature ectoines are receiving increasing attention by the scientific community because of their multiple applications. In fact, increasing commercial demand has led to a multiplication of efforts in order to improve processes for their production.

In this review, the importance of current and potential applications of ectoines as protecting agents for macromolecules, cells and tissues, together with their potential as therapeutic agents for certain diseases are analyzed and current theories for the understanding of the molecular basis of their biological activity are discussed. The genetic, biochemical and environmental determinants of ectoines biosynthesis by natural and engineered producers are described. The major limitations of current bioprocesses used for ectoines production are discussed, with emphasis on the different microorganisms, environments, molecular engineering and fermentation strategies used to optimize the production and recovery of ectoines. The combined application of both bioprocess and metabolic engineering strategies, allowing a deeper understanding of the main factors controlling the production process is also stated. Finally, this review aims to summarize and update the state of the art in ectoines uses and applications and industrial scale production using bacteria, emphasizing the importance of reactor design and operation strategies, together with the metabolic engineering aspects and the need for feedback between *wet* and *in silico* work to optimize bioproduction.

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* Corresponding author. Tel.: +34 968 367398; fax: +34 968 364148.

E-mail addresses: csonka@purdue.edu (L.N. Csonka), cargas@us.es (C. Vargas), mcanovas@um.es (M. Cánovas).

¹ Contributed equally to this work.

² Tel.: +34 954 553811; fax: +34 954 628162.

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1. Introduction to ectoines as compatible solutes

Halophilic microorganisms living in habitats of high ionic strength cope with hyperosmotic stress by changing the composition of membrane lipids and by regulating the intracellular concentration of low molecular weight solutes. As a result of the latter response, the cells are able to maintain proper osmotic balance under conditions of hyperosmotic stress, which is crucial to prevent the cell from leaking water, hence avoiding irreversible plasmolysis and dehydration and to generate turgor pressure within limits necessary for growth (Brown, 1990; Roessler and Muller, 2001). Cells use different strategies for the regulation of their internal osmolarity. One of these consists in the accumulation of inorganic salts, mainly KCl, to counterbalance the external salinity. This strategy has been adopted by the extremely halophilic haloarchaea, anaerobic moderately halophilic bacteria of the order *Haloanaerobiales* and the extremely halophilic bacterium *Salinibacter ruber* (Oren, 1999; Oren et al., 2002; Roberts, 2000). Because the enzymes and organelles of organisms that use this strategy need to function in environments of high ionic strength, they have evolved a number of extensive changes to make possible life under these extreme conditions. Foremost among these adaptive changes is that the enzymes of the KCl-accumulating organisms are much more acidic than orthologous proteins from mesophilic organisms (Dennis and Shimmin, 1997; Lanyi, 1974). This adaptation, however, is not useful for the colonization of habitats of moderate or low salinity since high intracellular salt concentration is always needed for correct protein folding and activity. Another, more versatile, strategy is the accumulation of “compatible solutes”, which generally are very soluble, low molecular weight, most either uncharged or zwitterionic organic molecules that are amassed in the cytoplasm. The latter types of solutes provide osmotic balance without interfering with the essential cellular processes and the normal metabolism and, since they have relatively little effect on the cytosolic ionic strength, no special adaptation of the intracellular systems (enzymes and organelles) is required (Brown, 1990; Oren, 1999). The level of compatible solute accumulation is set by the environmental osmolarity (Poolman and Glaesker, 1998). Upon a hypoosmotic shock, cells can restore the osmotic balance by releasing osmolytes via specific efflux systems, which are mechanosensitive channels different from the uptake systems (Morbach and Kramer, 2002). Therefore, this strategy enables organisms for rapid adaptation to an osmotically fluctuating environment by simply adjusting the internal solute pool to counteract the osmolarity of the surrounding environment. For this reason, this osmoadaptation strategy is widespread in nature, not only in Bacteria (Ventosa et al., 1998) and in some Archaea (Roessler and Muller, 2001), but also in Eukaryotes, including fungal, plant, animal and human cells (Burg and Ferraris, 2008; Yancey, 2005).

Compatible solutes fall into a few structural classes such as sugars (trehalose, sucrose), polyols (glycerol, sorbitol, manitol, α -glucosyl-glycerol, mannosyl-glycerol, and mannosyl-glyceramide), N-acetylated diamino acids (e.g., N-acetylglutaminylglutamine amide), betaines (like glycine betaine and derivatives), amino acids (proline, glutamate, glutamine, and alanine) and derivatives. The latter group includes ectoines (ectoine and hydroxyectoine). It has been demonstrated that most bacteria use an array of different solutes for osmotic balance, mainly depending on the duration of the osmotic stress, the level of salinity, the availability of substrates and osmolytes in the

surroundings or the carbon source used for the growth medium (Roberts, 2005).

1.1. Ectoine producing microorganisms, biosynthetic pathway and regulation

The capacity to synthesize ectoines is most widespread among α - and γ -Proteobacteria and Actinobacteridae, although it has been observed also in more limited number of β -, δ -, and ϵ -Proteobacteria, Firmicutes, and one Plantomycete (Table 1).

Ectoine can be considered to be a heterocyclic amino acid or as a partially hydrogenated pyrimidine derivative (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Galinski et al., 1985) (Fig. 1). It was discovered in the extremely halophilic phototrophic bacterium *Halorhodospira halochloris* (formerly *Ectothiorhodospira halochloris*) and characterized by ^{13}C -NMR spectroscopy, mass spectrometry and infrared spectroscopy (Galinski et al., 1985). This bacterium is able to grow at concentrations of up to 5 M NaCl (Raymond and Siström, 1969), which makes compatible solute accumulation compulsory for cell survival. The main compatible solutes produced were glycine betaine or trehalose (depending on the availability of nitrogen), although ectoine was also synthesized in the exponential growth phase. Ectoine is one of the most widely found compatible solutes throughout different halophilic and halotolerant microorganisms, from photosynthetic bacteria of the genus *Halorhodospira* to chemoheterotrophic bacteria, including γ -proteobacteria of the genera *Halomonas*, *Chromohalobacter*, *Vibrio*, *Pseudomonas* and *Marinobacter* (Roberts, 2005), actinobacteria including members of *Brevibacterium* and *Streptomyces* genera, firmicutes including several species of the genus *Bacillus* and closely related genera such as *Virgibacillus*, *Salibacillus* or *Halobacillus* (Kuhlmann and Bremer, 2002; Malin and Lapidot, 1996), and *Marinococcus halophilus* (Louis and Galinski, 1997), among others which accumulate ectoines (Table 1).

Hydroxyectoine was discovered in the actinomycin D producer *Streptomyces parvulus* (Inbar and Lapidot, 1988). This compatible solute is more common among Gram-positive halophilic/halotolerant bacteria including *Nocardioopsis* sp., *Streptomyces griseolus*, *Brevibacterium linens* or *M. halophilus* (Frings et al., 1995; Severin et al., 1992), but it is often synthesized at lower amounts together with ectoine in many other ectoine-producing species (Table 1). Despite being almost chemically identical to ectoine, hydroxyectoine seems to confer additional protective properties derived from its hydroxylated nature. Thus, whereas the main function of ectoine is to serve as an osmoprotectant, hydroxyectoine also seems to play an important role in heat stress protection. For instance, *Halomonas elongata*, *Chromohalobacter salexigens* and *Streptomyces griseus* accumulate hydroxyectoine in response to temperature upshift, and a *C. salexigens* mutant devoid of the main ectoine hydroxylase (the enzyme which converts ectoine into hydroxyectoine) is thermosensitive, providing evidence that hydroxyectoine functions as a thermoprotectant *in vivo* (García-Estépa et al., 2006; Malin and Lapidot, 1996; Wohlfarth et al., 1990). This property promoted the emergence of industrial processes specifically focused on hydroxyectoine production (Frings et al., 1995; Schiraldi et al., 2006).

The biosynthetic pathway of ectoines was biochemically established and studied in *H. halochloris*, *H. elongata* DSM 2581 (Peters et al., 1990) and *H. elongata* DSM 3043 (Canovas et al., 1997). The latter strain along

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