Biotechnology Advances 28 (2010) 782–801

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

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

ARTICLE INFO

Article history:
Received 20 April 2010
Received in revised form 18 June 2010
Accepted 22 June 2010
Available online 1 July 2010

Keywords:
Ectoine
Hydroxyectoine
Abiotic stress protection
Compatible solutes
Halophiles
Bioreactors
Bacterial milking

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.

Contents

1. Introduction to ectoines as compatible solutes . . . 783
   1.1. Ectoine producing microorganisms, biosynthetic pathway and regulation . . . 783
   1.2. Ectoines as carbon and energy sources . . . 787
2. Importance and uses of ectoines . . . 788
   2.1. Macromolecule protection . . . 788
      2.1.1. Effects on enzyme stability . . . 788
      2.1.2. Effects on DNA structure . . . 788
      2.1.3. Effect on protein structure . . . 789
   2.2. Biomedical potentials of ectoines . . . 789
      2.2.1. Protein stabilizing capacity of ectoines . . . 789
      2.2.2. Protective effects of ectoines in certain diseases . . . 790
      2.2.3. Cell protection capacity of ectoines . . . 790
      2.2.4. Skin protection capacity of ectoines . . . 791
   2.3. Understanding the activity of ectoines . . . 791
3. Production processes for ectoines . . . 792
   3.1. Ectoine . . . 793

* Corresponding author. Tel.: +34 968 367398; fax: +34 968 364148.
E-mail addresses: csonka@purdue.edu (L.N. Csonka), cvargas@us.es (C. Vargas), mcanovas@um.es (M. Cánovas).
[1] Contributed equally to this work.
[2] Tel.: +34 954 553811; fax: +34 954 628162.

0734-9750/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.biotechadv.2010.06.005

3.2. 5-Hydroxyectoine . . . 795
3.3. Heterologous ectoine producers . . . 795
4. Production systems . . . 796
5. Reactor systems . . . 796
6. Perspectives . . . 797
7. Conclusions . . . 798
Acknowledgements . . . 798
References . . . 798

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 Glaasker, 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 Sistrom, 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 *Nocardiopsis* 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* (Garcia-Estepa 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

Table 1
Microbial producers of ectoines: strains and conditions.

Microorganism	Compatible solutes	Salt concentration	Carbon source	References
Actinobacteria				
Brevibacterium epidermis DSM 20659	Ectoine	1 M (0 M downshock medium)	Monosodium glutamate + yeast extract	Onraedt et al. (2005)
Brevibacterium linens	Ectoine	1 M	L-glutamate (but "de novo" synthesis is the main way of production)	Bernard et al. (1993)
Brevibacterium sp. strain JCM 6894	Ectoine	2 M (osmotic upshock)	Glucose + yeast extract + Polypeptone	Nagata and Wang (2001)
Kocuria varians CCM3316	Ectoine, betaine, trehalose, hydroxyectoine	1.71 M	Yeast extract	Severin et al. (1992)
Nesterenkonia halobia DSM 20541^T	Ectoine, betaine, trehalose, hydroxyectoine	1.71 M	Yeast extract	Severin et al. (1992)
Nocardiopsis sp. A5-1	Ectoine, hydroxyectoine, trehalose	1.71 M	Glucose	Severin et al. (1992)
Streptomyces coelicolor A3 (2)	Ectoine, hydroxyectoine	0.5 M	Glucose	Bursy et al. (2008)
Streptomyces griseolus DSM 40067^T	Hydroxyectoine, betaine, trehalose	0.86 M	Yeast extract	Severin et al. (1992)
Firmicutes				
Bacillus alcalophilus DSM 485^T	Ectoine, glutamate	1 M	Glucose + Na-sesquicarbonate + amino acids and vitamins solution	Kuhlmann and Bremer (2002)
Bacillus agaradhaerens DSM 8721^T	Glutamate, ectoine. β-glutamate	1.3 M	Glucose	Bursy et al. (2007)
Bacillus clarkii DSM 8720^T	Glutamate, ectoine. hydroxyectoine, β-glutamate	1.8 M	Glucose	Bursy et al. (2007)
Bacillus halodurans DSM 497^T	Glutamate, ectoine. β-glutamate	1.6 M	Glucose	Bursy et al. (2007)
Bacillus mojavensis DSM 9205^T	Glutamate, proline, ectoine	1.5 M	Glucose	Bursy et al. (2007)
Bacillus pseudalcaliphilus DSM 8725^T	Glutamate, ectoine	1 M	Glucose + Na-sesquicarbonate	Bursy et al. (2007)
Bacillus pseudofirmus DSM 8715^T	Glutamate, proline, ectoine, alanine	1.6 M	Glucose	Bursy et al. (2007)
Gracilibacillus halotolerans DSM 11805^T	Glutamate, ectoine, hydroxyectoine	2 M	Glucose	Bursy et al. (2007)
Halobacillus halophilus DSMZ 2266^T	Ectoine, proline, glutamate glutamine	Increasing gradient 0.4–3 M	Glucose + vitamins solution	Saum and Muller (2008)
Halobacillus trueperi DSM 10404^T	Glutamate, ectoine, hydroxyectoine, N-ε-acetyl lysine	2.5 M	Glucose + vitamins solution + trace elements + amino acids	Bursy et al. (2007)
Marinococcus halophilus DSM 20408^T	Ectoine, hydroxyectoine	1.71 M	Glucose	Severin et al. (1992)
Marinococcus sp. M52	Hydroxyectoine	1.54 M	Fish peptone	Schiraldi et al. (2006)
	Hydroxyectoine, ectoine		Glucose	Severin et al. (1992)
	Hydroxyectoine, ectoine, glutamate	1.71 M	Glucose + fish peptone	Frings et al. (1995)
Salimicrobium albus DSM 20748^T	Ectoine, alanine, hydroxyectoine	1.71 M	Glucose	Severin et al. (1992)
Sporosarcina pasteurii DSM 33^T	Ectoine, glutamate	0.68 M (sucrose)	Glucose + urea + amino acids and vitamins solution	Kuhlmann and Bremer (2002)
Sporosarcina psycrophila DSM 3^T	Ectoine, glutamate	1 M	Glucose + amino acids and vitamins solution	Kuhlmann and Bremer (2002)
Virgibacillus marismortui DSM 12325^T	Glutamate, ectoine, hydroxyectoine	1.8 M	Glucose	Bursy et al. (2008)
Virgibacillus pantothenticus DSM 26	Ectoine, glutamate, hydroxyectoine	1 M	Glucose + amino acids and vitamins solution	Kuhlmann and Bremer (2002)
Virgibacillus salexigens DSM 11438	Ectoine, glutamate	3.4 M	Glucose + amino acids and vitamins solution	Kuhlmann and Bremer (2002)
Proteobacteria				
alpha-Proteobacteria				
Rhodovibrio salinarum BN 40	Betaine, ectoine	3.42 M	Phototrophic medium	Severin et al. (1992)
Rhodovulum sulfidophilum DSM 1374^T	Ectoine	1.71 M	Phototrophic medium	Severin et al. (1992)
gamma-Proteobacteria				
Chromohalobacter marismortui ATCC 17056	Ectoine, betaine	1.71 M	Yeast extract	Severin et al. (1992)
Chromohalobacter salexigens DSM 3043^T	Ectoine, hydroxiectoine, glutamate, glutamine	3 M	Glucose	Canovas et al. (1997)
	Ectoine, hydroxyectoine, glutamate, trehalose, NADA	2.5 M	Glucose	Garcia-Estepa et al. (2006)
Halomonas boliviensis DSM 15516^T	Ectoine, hydroxyectoine	2.58 M	Glucose, glutamate	Guzman et al. (2009)
Halomonas campisalis ATCC 700597	Ectoine, hydroxyectoine, glycine betaine	Increasing gradient 0–3 M	Glucose + yeast extract + trace element solution	Aston and Peyton (2007)
	Ectoine, hydroxyectoine, glycine betaine	Increasing gradient 0–3 M	Glucose + $NaNO_3$ + trace element solution	Aston and Peyton (2007)

Table 1 (*continued*)

Microorganism	Compatible solutes	Salt concentration	Carbon source	References
Proteobacteria				
gamma-Proteobacteria				
Halomonas elongata ATTC 33173^T	Ectoine, glutamate, glycine betaine, alanine, hydroxyectoine	1.3–1.7–4.3 M	Glucose	Wohlfarth et al. (1990)
	Ectoine, hydroxiectoine	1.71 M	Glucose	Severin et al. (1992)
	Ectoine		Glucose	Peters et al. (1990)
	Ectoine, trehalose, betaine	Increasing gradient 0.51≥4.28 M	Glucose	Maskow and Babel (2001)
Halomonas elongata DSM 142	Ectoine, glutamate, alanine, NADA, hydroxyectoine	2.56 M (batch medium), 0 M (downshock medium)	Glucose	Sauer and Galinski (1998)
Halomonas elongata K63	Ectoine, alanine, NADA, hydroxyectoine	2.56 M	Glucose	Ono et al. (1998)
Halomonas halmophila CCM 2833^T	Ectoine, glutamate, alanine	1.71 M	Glucose	Wohlfarth et al. (1990)
	Ectoine, hydroxyectoine	1.71 M	Glucose	Severin et al. (1992)
Halomonas halophila CCM 3662^T	Ectoine, glutamate	1.71 M	Glucose	Severin et al. (1992)
Halomonas halodenitrificans DSM 735^T	Ectoine	1.71 M	Glucose	Severin et al. (1992)
Halomonas variabilis DSM 3051^T	Hydroxyectoine, betaine, ectoine, trehalose	1.71 M	Yeast extract	Severin et al. (1992)
Halorhodospira abdelmalekii DSM 2110^T	Betaine, ectoine, trehalose	3.42 M	Phototrophic medium	Wohlfarth et al. (1990)
Halorhodospira halochloris DSM 1059^T	Ectoine		Acetate, bicarbonate (grown photosynthetically)	Peters et al. (1990)
	Betaine, ectoine, trehalose	3.42 M	Phototrophic medium	Wohlfarth et al. (1990)
Halorhodospira halophila DSM 244^T	Betaine, ectoine, trehalose	3.42 M	Phototrophic medium	Wohlfarth et al. (1990)
Marinobacter hydrocarbonoclasticus	Ectoine, betaine, glutamate, beta-glutamate	1.5 M	Eicosane (sole carbon source)	FernandezLinares et al. (1996)
Pantoea agglomerans strain CPA-2	Ectoine, glycine betaine	1.2 M	Sucrose + Yeast extract	Canamas et al. (2007)
Pseudomonas halophila DSM 3050^T	Betaine, hydroxyectoine, ectoine	1.71 M	Yeast extract	Severin et al. (1992)
Pseudomonas halosaccharolytica CCM 2851	Ectoine, alanine, hydroxyectoine	1.71 M	Glucose	Severin et al. (1992)
Thioalkalibacter halophilus DSMZ 19224	Ectoine, glycine betaine	3 M	Glucose	Banciu et al. (2008)
Vibrio alginolyticus DSM 2171^T	Ectoine, alanine	1.71 M	Glucose	Severin et al. (1992)
Vibrio cholerae O139 strain MO10	Ectoine	0.5 M	Mixture of amino acids	Pflughoeft et al. (2003)
Vibrio costicola CCM 2811	Ectoine, betaine	1.71 M	Yeast extract	Severin et al. (1992)
Vibrio fischeri DSM 507	Ectoine, glutamate, betaine	0.86 M	Complex	Schmitz and Galinski (1996)
Vibrio fischeri DSM 7151	Glutamate, ectoine, betaine	0.86 M	Complex	Schmitz and Galinski (1996)
Vibrio harveyi DSM 2165	Ectoine, GABA, glutamate, alanine	0.86 M	Glycerol	Schmitz and Galinski (1996)
Vibrio harveyi DSM 6904	Ectoine, glutamate	0.86 M	Glycerol	Schmitz and Galinski (1996)
Vibrio parahaemolyticus RIMD2210633	Ectoine	1 M	Mixture of amino acids	Naughton et al. (2009)

with *H. elongata* ATCC 33174, were reclassified into the genus *Chromohalobacter*, as the new species *C. salexigens* DSM 3043 and ATCC 33174 (Arahal et al., 2001). For the sake of clarity, all of them will be referred as *C. salexigens* along this review, in order to differentiate them from the rest of *H. elongata* strains, which were not reclassified. The ectoine biosynthetic pathway starts with the phosphorylation of L-aspartate and shares its first two enzymatic steps with the biosynthesis

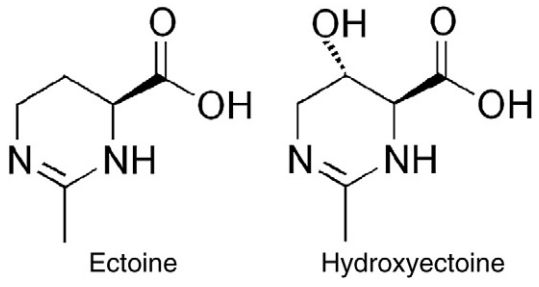

Fig. 1. Chemical structures of ectoines.

of amino acids of the aspartate family: the conversion of L-aspartate into L-aspartate–phosphate by the aspartate kinase (Ask), and the synthesis of L-aspartate-beta-semialdehyde from L-aspartate-phosphate, catalyzed by the L-aspartate-beta-semialdehyde dehydrogenase (Asd). From this general intermediate, the specific route for ectoine synthesis occurs in three enzymatic steps. First, L-aspartate-beta-semialdehyde is converted into L-diaminobutyric acid (by the enzyme L-diaminobutyric acid transaminase, EctB or ThpB), and is subsequently acetylated to N-γ-acetyldiaminobutyric acid (NADA) by the enzyme L-diaminobutyric acid acetyl transferase (EctA or ThpA). Finally, the cyclic condensation of NADA leads to the formation of ectoine through the activity of the enzyme ectoine synthase (EctC or ThpC) (Fig. 2). The main biosynthetic route to hydroxyectoine involves hydroxylation of ectoine by ectoine hydroxylase (EctD or ThpD), which has been characterized in *C. salexigens* and *Streptomyces chrysomallus* (Garcia-Estepa et al., 2006; Grammel, 2000) (Fig. 2).

In addition, a secondary route for hydroxyectoine synthesis without the involvement of ectoine, branching off the ectoine precursor NADA, has been suggested for *C. salexigens* (Fig. 2). However, the first enzyme(s) for this alternative pathway seem to show very little affinity for NADA, since

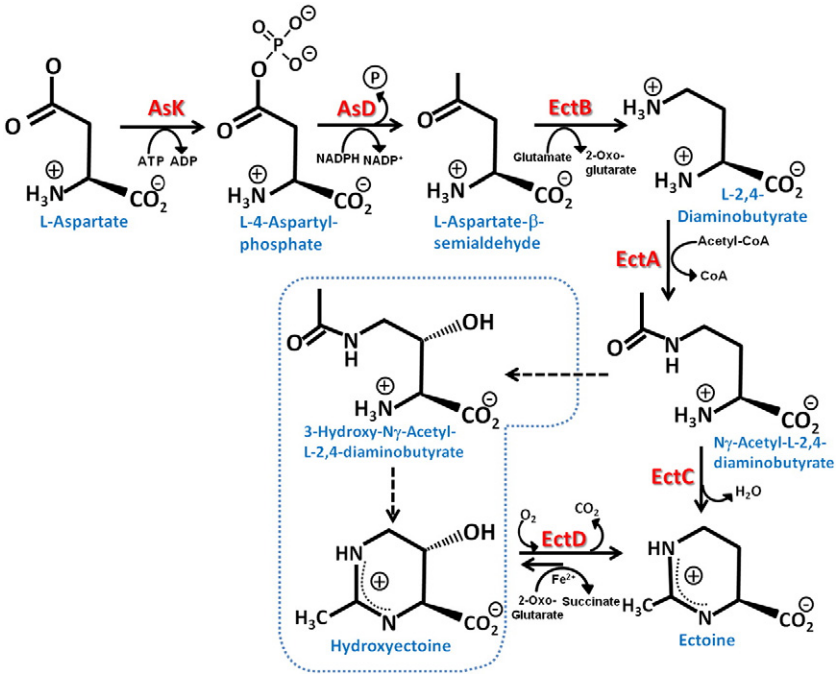

Fig. 2. Pathway for the synthesis of ectoines in *C. salexigens*.

an *ectC* mutant, affected in the ectoine synthase gene, accumulates large amounts of the ectoine precursor. This finding suggests that either the enzyme(s) of the alternative pathway for hydroxyectoine synthesis hydroxylated NADA in a non-specific way, or that the alternative pathway functions under environmental conditions different from those assayed so far (Canovas et al., 1999).

Genes encoding the enzymes for the biosynthesis of ectoines have been identified in 50 bacterial genomes and in one archaeon, *Nitrosopumilus maritimus* (Lo et al., 2009). In these organisms, the *ectABC* genes are in almost all cases arranged as an operon, but there are rare (so far) exceptions, which might be mentioned. For instance, the γ-proteobacterium *Nitrosococcus oceani* carries *ectABD*, and *ectCask* (*ask* encoding aspartate kinase) within two separated transcriptional units. The α-proteobacterium *Marinobacter aquaeolei* is exceptional in having *ectABask* in one transcriptional unit, three copies of *ectC* and two copies of *ectD*, all scattered within its chromosome. Redundancy of *ectD* also occurs in the γ-proteobacterium *C. salexigens*, which carries two functional copies of *ectD* (*ectD* and *ectE*, M. Reina-Bueno and C. Vargas, unpublished data). In a subset of the α-, β-, and γ-*Proteobacteria*, there is a putative transcriptional regulatory gene (*ectR*) upstream of *ectA*, which is transcribed in the same direction as *ectABC* in β-*Proteobacteria*, and the opposite direction in α- and γ-*Proteobacteria* (Lo et al., 2009). In α-, β-*Proteobacteria*, *Actinobacteridae*, in a subset of *Firmicutes*, and in *N. maritimus*, *ectABC* are followed by the *ectD* gene as part of the same operon. This organization can also be found in some γ-*Proteobacteria*, but in a number of members of this subdivision, notably including *C. salexigens*, *ectD* is distant from the *ectABC* operon. Finally, in α- and some γ- and δ-*Proteobacteria ask* lays immediately downstream of and is co-transcribed with the *ectABC*(*D*) operon. The chromosomal position of the *ask-ect* gene suggests that might be co-regulated transcriptionally with the *ectABC*(*D*) genes (Lo et al., 2009).

Frequently, organisms have two or more aspartyl phosphate forming isoenzymes, whose allosteric regulation suggests that they have different dedicated functions in the synthesis of various biosynthetic products derived from aspartate (threonine, methionine, lysine, and ectoine). However, *C. salexigens* is an outlier, having only one aspartate kinase, which in addition is not near the *ectABC* operon in this organism. The control of the aspartate kinase has not been studied in *C. salexigens*, but because this enzyme carries out the first reaction of a divergent pathway that leads to the synthesis of multiple biosynthetic precursors as well as ectoine, it will be both interesting and important to elucidate its transcriptional and allosteric control in this and other ectoine-producing organisms. *C. salexigens* DSM 3043 growth is inhibited by threonine in glucose-minimal medium, which is consistent with the possibility that this amino acid might be a feedback inhibitor of aspartate kinase, although the growth inhibition was not relieved by lysine and/or methionine or glycine betaine (which could supplant ectoine as a compatible solute) (K. O'Connor and L. N. Csonka, unpublished data). If the sole aspartate kinase of *C. salexigens* is subjected to allosteric regulation by its biosynthetic end-product(s), it might be possible to increase the production of ectoines by overproducing an allosteric feedback-insensitive mutant form of this enzyme.

Knowledge on the regulation of ectoine synthesis is important in order to generate engineered strains that are improved in ectoine production for prospective industrial use. In Gram-negative bacteria, most regulatory studies have been carried out in *C. salexigens* (Fig. 3). On the one hand, physiological data indicate that the levels of ectoine and hydroxyectoine are maximal during the stationary phase and that ectoine accumulation is up-regulated by salinity, whereas hydroxyectoine accumulation is up-regulated by both high salt and high temperature (Garcia-Estepa et al., 2006). This regulation occurs, at least in part, at the transcriptional level. S1 protection assays and transcriptional fusions with the reporter gene *lacZ* demonstrated that the ectoine synthesis genes (*ectABC*) can be expressed from two promoter regions. One is located upstream of *ectA* and it is composed of four putative promoters (*PectA1–4*) and the second one is an internal promoter located upstream of *ectB* (*PectB*). Expression of *PectA–lacZ* and *PectB–lacZ* transcriptional fusions are maximal during stationary phase (in agreement with the physiological data) but they are also transcribed at reduced levels at low salinity, suggesting that *ectABC* may be partially constitutive. *PectA* expression is osmoregulated (in an *E. coli* background), and the S1 protection assays suggest that *PectA1*, *PectA3* and *PectA4* may be the osmoregulated promoters within the *PectA* region. Expression of *PectA*, and especially, *PectB* is induced by continuous growth at high temperature, and repressed in the presence of osmoprotectants (betaine and ectoine), the DNA gyrase inhibitor nalidixic acid and an excess of iron (only *PectA*) (Calderon et al., 2004). These data suggest that transcription of ectoine synthesis genes involves

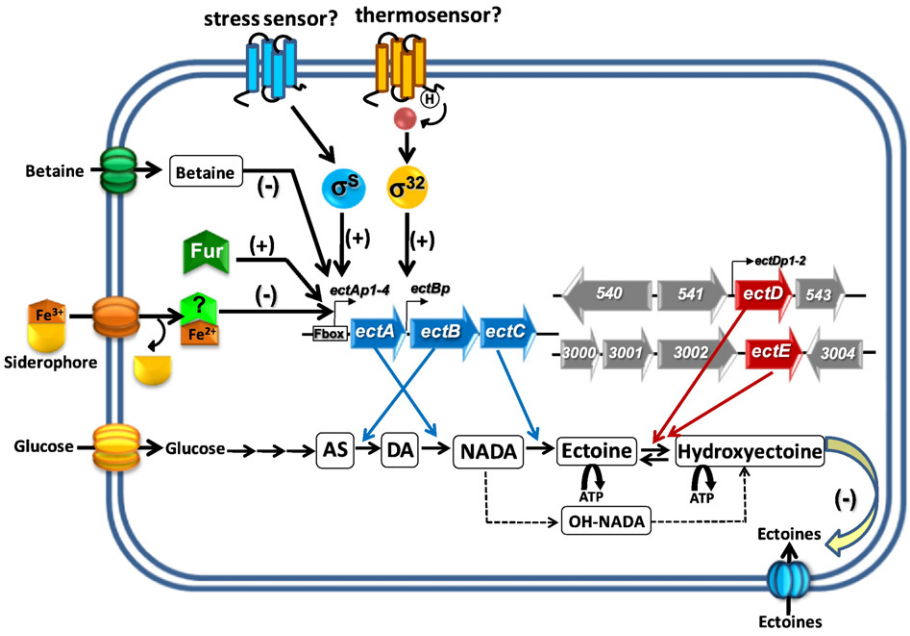

Fig. 3. Accumulation of ectoines in *C. salexigens* and transcriptional control mechanisms.

multiple promoters that allow the system to be regulated by a number of environmental factors, including salinity, temperature, external osmoprotectants and iron. Ensuring appropriate expression in response to changing environments needs the involvement of a number of transcription factors belonging to different regulatory pathways. The *in silico* analysis of the −10 and −35 sequences of the regions upstream of *ectA*, *ectB* and *ectD* showed that *PectA1* and *PectA2* overlap with putative recognition sites of both the main vegetative factor σ^{70} (Calderon et al., 2004) and the iron homeostasis regulator Fur (Argandoña et al., 2010), whereas *PectA3* is similar to σ^{S}-dependent promoters and *PectB* may be recognized by the heat stress factor σ^{32} (Calderon et al., 2004).

Although temperature induction of *PectA* and *PectB* expression initially suggested that, in addition to osmoprotection, ectoine might have a physiological role in thermoprotection of *C. salexigens* (Calderon et al., 2004) ectoine levels do not increase in response to temperature (Garcia-Estepa et al., 2006). One explanation for this is that at high temperature ectoine is rapidly converted to hydroxyectoine by *C. salexigens*, therefore reducing the levels of ectoine. This is consistent with high temperature induction of the ectoine synthesis genes, since ectoine is the precursor of hydroxyectoine. In agreement with the general assumption that transport of compatible solutes is preferred over the synthesis, because the latter is energetically less favourable (Oren, 1999), *C. salexigens* cells growing with betaine do not accumulate ectoine(s) at any salinity tested (Calderon et al., 2004). As betaine only represses partially the expression of *PectA* and *PectB*, the existence of a post-transcriptional control mechanism that might operate at the level of enzyme activity may be inferred. However, other alternative mechanisms such as an increased efflux of ectoine in the presence of betaine cannot be ruled out.

In Gram-positive bacteria, regulation of ectoine synthesis was addressed in *Salibacillus pasteurii* (Kuhlmann and Bremer, 2002) and *M. halophilus* (Bestvater and Galinski, 2002; Louis and Galinski, 1997). Primer extension analysis showed that the *ectABC* genes of *Salibacillus pasteurii* are expressed from a single osmoregulated promoter whose −10 and −35 sequences resembled the consensus sequences of promoters recognized by the main vegetative sigma factor σ^{A} (orthologue of Gram-negative σ^{70}). However, in *M. halophilus*, computer-assisted analysis of the DNA upstream of *ectABC* revealed the presence of a putative binding site for σ^{B} (orthologue of Gram-negative σ^{S}), as the most promising candidate for transcription regulation in this organism (Bestvater and Galinski, 2002; Louis and Galinski, 1997). Very interestingly, a transcriptional fusion of this promoter region (referred to as *ect*Up) with the reporter gene *gfp* was not only expressed but also osmotically induced in *E. coli* (Bestvater and Galinski, 2002; Louis and Galinski, 1997). The presence of sequences at the promoter region that might be potentially recognized by the *E. coli* sigma factors σ^{70} or σ^{S} may explain this heterologous expression. Similarly to σ^{S} in Gram-negative bacteria, σ^{B} controls the general stress response in Gram-positive bacteria, and it is partially responsible for the transcriptional control of some osmoregulated genes, such as *opuE* and *opuD* from *Bacillus subtilis*, encoding the proline and glycine betaine uptake systems, respectively (Bremer and Kramer, 2000). So far, the involvement of σ^{A} and/or σ^{B} in the control of ectoine synthesis in any of these two bacillus-like microorganisms has not been experimentally shown.

Ectoine transport and its regulation have been extensively studied by HJ Kunte and co-workers in the Gram-negative *H. elongata* DSM 2581 and the Gram-positive *M. halophilus* (see Kunte (2006) for a review). In *H. elongata*, these authors found a transcriptional unit containing three genes, *teaABC*, which showed to be essential for ectoine uptake (Grammann et al., 2002). *In silico* analysis predicted TeaABC to belong to the family of TRAP transporters, consisting of two putative transmembrane proteins (TeaB and TeaC) and a periplasmic ectoine-binding protein (TeaA) (Grammann et al., 2002; Tetsch and Kunte, 2002). *C. salexigens* genome carries orthologues to *H. elongata teaABC*, but arranged differently (*teaA* in one strand, followed by *teaBC* in the opposite strand). We have experimental evidence that this system is the main responsible for the uptake of ectoines in *C. salexigens* (J. Rodriguez-Moya and C. Vargas, unpublished data). On the other hand, the *M. halophilus* ectoine transporter, EctM, was predicted to be an osmoregulated hydrophobic, 525-residue protein, which shared significant identity to betaine–carnitine–choline transporters (BCCTs). This protein was suggested to have a high affinity for ectoine (Vermeulen and Kunte, 2004).

1.2. Ectoines as carbon and energy sources

In addition to stress protection, one important secondary function of compatible solutes may be to serve as carbon, energy or nitrogen sources, either as intracellular reserves or as external sources made available to other organisms by release into the environment upon death or hypoosmotic shock of the producing strains. Carbohydrates such as glycerol, glucosylglycerol, trehalose, sucrose and amino acids such as

proline, glutamate and alanine, which are common compatible solutes in a wide range of Gram-positive and Gram-negative bacteria, actinomycetes and micro-algae, can be used as carbon, nitrogen and energy sources by a vast array of heterotrophic and phototrophic microorganisms (Welsh, 2000). In contrast, catabolism of ectoines (ectoine and hydroxyectoine) seems to be much more infrequent. Thus, the halotolerant species *Sporosarcina pasteurii* synthesizes and accumulates ectoine but cannot degrade ectoine or hydroxyectoine (Kuhlmann and Bremer, 2002). Similarly, *E. coli* can use ectoine as an osmoprotectant and hydroxyectoine both as osmo- and thermotoprotectant by transport from the medium, but cannot catabolize them as carbon or nitrogen sources (Jebbar et al., 1992; Malin and Lapidot, 1996).

There are, however, some bacteria able to use ectoine(s) for catabolic purposes. Thus, ectoine is metabolized, rather than accumulated, by *Sinorhizobium meliloti* under high osmolarity conditions, where it seems to enhance the synthesis of endogenous compatible solutes (Talibart et al., 1994). *H. halochloris*, the phototrophic sulphur bacterium in which ectoine was discovered, can degrade its internal ectoine pool during nitrogen starvation and replace it by trehalose, thus liberating nitrogen for cell growth (Galinski and Herzog, 1990). In addition, Manzanera et al. (2002) reported that *Pseudomonas putida* is able to internalise and degrade hydroxyectoine for catabolic purposes, but a similar role of ectoine was not investigated.

The biochemical pathway(s) leading to ectoine (and hydroxyectoine) degradation remains unknown. One important step towards the elucidation of these catabolic routes was the identification by Jebbar et al. (2005) of an ectoine-induced operon involved in the uptake and catabolism of ectoine in *S. meliloti* (*ehuABDC–eutABCD*). On the basis of sequence homologies, *eutABCDE* were found to encode enzymes with putative functions in ectoine metabolism. In addition, analysis of the properties of a *eutA* mutant unequivocally demonstrated that at least some of the *eut* genes are involved in ectoine degradation by *S. meliloti*. However, ectoine catabolism was not completely abolished in the mutant, suggesting that *S. meliloti* contains other genes encoding ectoine degradation enzymes.

Ectoine and hydroxyectoine serve as carbon sources for *C. salexigens* exclusively when grown under optimal osmolarity conditions (Vargas et al., 2006). In agreement with this, the transport of ectoine was maximal at the optimal salt concentration. In this microorganism, ectoine utilization was only partially down-regulated by glucose, suggesting the existence of at least two systems involved in ectoine catabolism in *C. salexigens*. This situation might be similar to that found in *S. meliloti*, where in addition to EutABCDE there is evidence for at least another ectoine-degrading pathway. Within the draft sequence of the *C. salexigens* genome (available at http://genome.jgi-psf.org/mic_home.html) we have found a cluster of 11 genes, all oriented in the same direction, which may encode orthologues to EutB, EutC, EutD, and EutE ("E values" in BLASTP [http://www.ncbi.nlm.nih.gov/BLAST] ranging from 3e-72 to e-140). However, we did not find statistically significant matches to EutA, and the *eutBCDE* genes were organized differently, and apart from the *ehu* transport system found in *S. meliloti* (C. Vargas, unpublished results).

2. Importance and uses of ectoines

2.1. Macromolecule protection

An additional role for compatible solutes in general is that they can mitigate deleterious effects of heat stress, freezing, drying, high salinity, oxygen radicals, radiation, urea and other denaturing agents on the integrity of proteins, nucleic acids, biomembranes and even whole cells (da Costa et al., 1998; Lentzen and Schwarz, 2006). Among the different compatible solutes so far investigated, ectoines have shown to display the most powerful stabilizing properties (Lippert and Galinski, 1992).

2.1.1. Effects on enzyme stability

The stabilizing properties of ectoines have been assayed in a variety of different enzymatic processes (Table 2). The thermodynamic aspects of protein stabilization by hydroxyectoine were studied by using differential scanning calorimetry and bovine ribonuclease A (Knapp et al., 1999). The significant stabilization of RNase A by hydroxyectoine makes it an interesting stabilizer in biotechnological processes in which enzymes are applied in the presence of denaturants or at high temperature (Knapp et al., 1999). Hydroxyectoine is also able to protect lactate dehydrogenase (LDH) from metal-catalyzed oxidation and against oxidation by hydrogen peroxide (Andersson et al., 2000), indicating that the protection is independent of the source of oxidative damage.

LDH and phosphofructokinase are highly susceptible to loss of activity by freeze-thawing, heating and freeze-drying. However, the activities of both enzymes were protected by ectoines under these treatments (Lippert and Galinski, 1992). The structural changes of LDH under conditions of freeze-thawing and urea treatment and the protection of the enzyme by ectoines were observed and it was proposed that the ectoines lead to a more compact conformation of the enzyme by modulating solvent properties (Göller and Galinski, 1999), supporting the hypothesis of "preferential exclusion of solutes from the protein surface" (Arakawa and Timasheff, 1985) (see Section 2.3.).

The polymer cyanophycin is a reserve material for nitrogen and energy of biotechnological interest (Joentgen et al., 2001) because the oligo-arginyl polyaspartic acid obtained by its degradation may be used as a biodegradable substitute in various technical products and processes (Schwamborn, 1998). Ectoine has been used to stabilize a new heterologous cyanophycin synthetase for *in vitro* cyanophycin and polyamide production on a technical scale (Hai et al., 2002). The activity of phytase, a phosphomonoesterase that hydrolyzes phytate and releases inorganic phosphate during feed pelleting (Keshavarz, 2000; Stahl et al., 2000), usually decreases due to the high temperature reached in this process (Ullah, 1988). Interestingly, ectoine markedly stabilizes the catalytic capacity of phytase at high temperature, showing a seven fold increase, if compared to that without ectoine at high temperature (Zhang et al., 2006).

2.1.2. Effects on DNA structure

It was shown that ectoines induce a change in the structure of DNA such that many restriction endonucleases were no longer able to cleave it (Malin et al, 1999) (Table 2). Thus, addition of compatible solutes during a PCR reaction may change DNA conformation in a way that influences primer annealing and binding of the polymerase. It was confirmed that ectoine increases the thermal stability of DNA polymerases at elevated temperatures and diminishes the melting temperature of double stranded DNA (Lapidot et al., 1995). This second effect is greater for GC-rich templates than for GC-poor templates, being remarkable for homoectoine, a new synthetic derivative of ectoine (Table 2). Besides, homoectoine enhances the specificity of PCRs to 100%, avoiding the contaminating products supplemented in ectoine PCRs at some concentrations (Schnoor et al., 2004). Because of these properties, ectoines can enhance the efficiency of polymerase chain reactions with GC-rich templates but also to improve primer extension or sequencing methods (Lapidot et al., 1995).

The optimization of the microarray workflow, including the hybridization step is a primary target for the development of more efficient protocols. Thus, low millimolar concentrations of hydroxyectoine, potassium diglycerol phosphate and potassium mannosylglycerate reduced DNA microarray background (Table 2) and improved hybridization efficiency (Mascellani et al., 2007). In recent years it became clear that transcriptional regulation and recombination not only requires the interaction of sequence-specific proteins but also often depends on the association of DNA binding proteins without sequence-specificity (Azam and Ishihama, 1999; Rimsky et al., 2001; Schroder and Wagner, 2002). It was shown that the generalized DNA binding protein

Table 2
Importance and uses of ectoines.

Effect	Compound	References
Macromolecules protection		
Protection of recombinant proteins against degradation, agregation, misfolding and freezing	Hydroxyectoine	Barth et al. (2000)
Thermostability of cyanophycin synthetase	Ectoine	Hai et al. (2002)
Enzyme protection against heating freezing and drying.	Hydroxyectoine, ectoine	Lippert and Galinski (1992)
Thermostability of phytase (90 °C)	Ectoine	Zhang et al. (2006)
Protection of antibodies against proteolytic degradation	Ectoine	Bersch et al. (2000)
Protection against freeze–thaw stress of immunotoxins	Hydroxyectoine	Barth et al. (2000)
Effective lowering of DNA melting temperature	Ectoine	Lapidot et al. (1999)
PCR enhancer	Homoectoine	Schnoor et al. (2004)
Improve the quality of DNA microarrays	Hydroxyectoine	Mascellani et al. (2007)
Arresting of the cleavage site of plasmid and lambda DNA by Type II endonucleases	Hydroxyectoine	Malin et al. (1999)
Protection of oxidative protein damage (LDH)	Hydroxyectoine	Andersson et al. (2000)
Stabilization of the complex LRP and H-NS in transcription regulation	Ectoine	Pul et al. (2007)
Increasing stability and melting temperature of RNase A	Hydroxyectoine	Knapp et al. (1999)
Protection of LDH against heat, urea and freeze-thawing	Hydroxyectoine, ectoine	Göller and Galinski (1999)
Cell membrane protection against surfactant agent	Ectoine	Bunger (1999), Buenger et al. (2001), Graf et al. (2008)
Optimization of protein crystallization conditions for X-ray crystallography	Ectoine	Harjes et al. (2004)
Potential therapeutic uses		
Protection against nanoparticle-induced neutrophilic lung inflammation	Ectoine	Sydlik et al. (2009)
Protection small bowel from cold ischemia and subsequent warm *in vitro* reperfusion injury	Ectoine	Wei et al. (2009)
Protection of neuronal cells from polyglutamine-induced toxicity in the Machado–Joseph disease	Ectoine	Furusho et al. (2005)
Inhibition of the aggregation and neurotoxicity of Alzheimer's beta-amyloid	Ectoine/hydroxyectoine	Kanapathipillai et al. (2005)
Inhibition of insulin amyloid formation	Ectoine	Arora et al. (2004)
Protection for zymogens against proteolysis	Ectoine	Kolp et al. (2006)
Inhibit binding of a Tat-like, arginine-containing peptide, to HIV TAR RNA *in vitro*	Hydroxyectoine	Lapidot et al. (1995)
Inhibit aggregation and neurotoxicity of prion peptide 106–206	Ectoine	Kanapathipillai et al. (2008)
Stabilization of retroviral vectors for gene therapy	Hydroxyectoine	Cruz et al. (2006)
Cell protection		
Protection against dessication on *Pseudomonas putida*	Hydroxyectoine	Manzanera et al. (2002)
Protection against dessication on *E. coli*	Hydroxyectoine	Manzanera et al. (2004a)
Protection against plastic encapsulation of *E. coli* and *P. putida*	Hydroxyectoine	Manzanera et al. (2004b)
Promoting effect on the ethanol fermentation by *Zymomonas mobilis*	Ectoine	Zhang et al. (2008)
Implicated in detoxification of phenol in *Halomonas*	Ectoine	Maskow and Kleinsteuber (2004)
Preservation of the respiratory activity on *Escherichia coli* (*in vivo*)	Ectoine	Nagata et al. (2002)
Osmoprotection of lactic acid bacteria	Ectoine	Baliarda et al. (2003)
Induction of thermotolerance in *E. coli*	Hydroxyectoine	Malin and Lapidot (1996)
Stabilization of *E. coli* during drying and storage	Ectoine, hydroxyectoine	Louis et al. (1994)
Salt tolerance in ectoine-transformed tobacco plants	Ectoine	Moghaieb et al. (2006)
Salt tolerance in ectoine-transformed tobacco plants cells	Ectoine	Nakayama et al. (2000)b
Skin protection		
In vivo antiageing skin protection in humans	Ectoine	Heinrich et al. (2007)
In vivo protection against dehydration on the skin and *in silico* models for explaining it.	Ectoine	Graf et al. (2008)
In vitro antiageing skin protection in humans, mitochondrial DNA protection and inhibition of the inflammatory response mediated by ceramides.	Ectoine	Buenger and Driller (2004)
Induction of heat shock proteins and mediating in the proinflammatory response in the human keratinocytes	Ectoine	Buommino et al. (2005)
In vitro photoprotection of visible light	Ectoine	Botta et al. (2008)
In vivo moisturizer agent of human skin	Ectoine	Motitschke et al. (2000)
In vivo protection UV-induced degradation Langerhans cells	Ectoine	Beyer et al. (2000)
In vitro inhibition of the formation of SBCs and *in vivo* protection langerhans cells	Ectoine	Buenger et al. (2001) and Pfluecker et al. (2005)
Block of UVA-induced ceramide release in human keratinocytes	Ectoine	Grether-Beck et al. (2005)
In vivo protection against dehydration on the skin by surfactants	Ectoine	Bunger (1999)

H-NS stimulates LRP (leucine response protein) binding without being present in the final complex (Table 2). The presence of the osmolyte ectoine, which ensures that single-cell organisms maintain the proper level of hydration, also stimulates LRP binding (Pul et al., 2007).

2.1.3. Effect on protein structure

Insoluble or misfolded overexpressed proteins can often be partially denatured and refolded in the presence of osmolytes (Table 2). A specific example is the use of hydroxyectoine in the freeze–thaw purification process to enhance the yield of folded functional cytotoxic proteins overexpressed and redirected to the periplasm of *E. coli* (Barth et al., 2000). Ectoine was also shown to protect proteins from proteolysis by trypsin and trypsinogen preserving its activity during incubation. Then, ectoines could function as promising additives whenever the use of protease inhibitors should be avoided. This may be the case for investigations on proteases as targets for the development of drugs (Kolp et al., 2006).

2.2. Biomedical potentials of ectoines

2.2.1. Protein stabilizing capacity of ectoines

Recently, there has been an increasing recognition of diseases associated with the misfolding of proteins (Dobson, 2003). In an important subgroup of misfolding diseases, protein aggregation leads

to the formation of highly regular aggregates termed amyloids. This phenomenon plays a key role in the development of diseases like Alzheimer's disease (AD) and spongiform encephalopathies (Harper and Lansbury, 1997; Murphy, 2002) (Table 2). It was shown that ectoine is a very effective inhibitor of amyloid formation decreasing both its initiation and elongation phase, in a study that used the formation of insulin amyloid *in vitro* as a model system (Arora et al., 2004).

Ectoines have been reported to be potential candidates of anti-amyloid therapeutics for treating Alzheimer's disease because they strongly inhibit the Aβ42 amyloid formation *in vitro* and reduce the toxicity to human neuroblastoma cells (Kanapathipillai et al., 2005) (Table 2). Prions are infectious particles that cause transmissible spongiform encephalopathies in animals and humans (Aguzzi and Polymenidou, 2004). They aggregate into protease-resistant amyloid fibrils, thereby inducing neuronal cell death by apoptosis, and causing proliferation and hypertrophy of cultured glia (Forloni et al., 1993; Pan et al., 1993). Neuroblastoma cells co-incubated with ectoine together with prions showed higher survival rate compared to control samples inhibiting prions amyloid formation and reducing their cytotoxicity (Kanapathipillai et al., 2008).

Protein misfolding is also a key event in the pathogenesis of polyglutamine diseases such as Machado–Joseph disease (MJD) (Table 2). Misfolding of the protein fragment containing an expanded polyglutamine stretch is considered significant in producing aggregates and cell death (Kakizuka, 1998; Paulson, 1999; Perutz, 1999). Previous studies suggest that nuclear inclusion is not a causative agent of the cytotoxicity, being instead protective by sequestering the toxic polyglutamine-expanded protein (Ferrigno and Silver, 2000). Ectoine reduces apoptotic features by reducing the total amount of aggregates and changing its intracellular distribution. Thus, it decreases cytotoxicity increasing frequency of nuclear inclusions (Furusho et al., 2005).

2.2.2. Protective effects of ectoines in certain diseases

Ectoine administration inhibited nanoparticle-induced signaling, which is known to be responsible for proinflammatory reactions in lung epithelial cells (Sydlik et al., 2009) (Table 2). The inhalation of nanoparticles has been identified as a major driving hazard in the ambient air of modern industrialized societies. It is able to induce lung inflammation, the central pathogenic mechanism responsible for a multitude of organic and systemic diseases (Donaldson et al., 2005). The lung, as the primary target organ for particle-induced inflammation, is severely damaged by the constant recruitment and activation of inflammatory cells. Lung diseases like emphysema, chronic obstructive pulmonary disease, fibrosis and cancer (Bringardner et al., 2008; Macnee, 2007) as well as systemic disorders on the level of the cardiovascular and the immune system (Peters et al., 2004) occur as a consequence of these events.

Ischemia and subsequent reperfusion injury (I/R) remains a major obstacle to successful small bowel transplantation (Grant et al., 1990). It is generally accepted that I/R injury is the underlying pathophysiological mechanism for mucosal damage (Kong et al., 1998). Although intestinal mucosa has high regenerative ability, it is demonstrated that morphological recovery of the injured ileal mucosa after 24 h cold storage requires at least 1 month. This prolonged damage surely contributes not only to acute graft rejection, but also to various postoperative complications such as bacterial translocation, endotoxin absorption and long-lasting malnutrition (Zou et al., 2005). Ectoine effectively protects ileal mucosa and muscularis by diminishing ceramide as a mediator of apoptosis and oxidative stress consistent with simultaneous mitochondrial damage improving mucosal barrier function (Wei et al., 2009) (Table 2).

Another example is the replication of human immunodeficiency virus (HIV), which is critically dependent on two viral regulatory proteins, Tat and Rev. Tat is required in the early viral life cycle for efficient transcription of the viral genome. Tat acts by binding to an RNA loop structure, the trans-acting responsive element (TAR), located at the 5′ end of the viral mRNA (Cordingley et al., 1990; Dingwall et al., 1990; Roy et al., 1990). It has been reported that ectoine inhibits the interaction of Tat peptide with TAR RNA being ~10^6 times more tightly bound. Besides, the uptake of [^{14}C] ectoines by both HeLa cells and human fibroblast cell line under normal cell growth conditions facilitates their consideration as potential antiviral drugs (Lapidot et al., 1995) (Table 2).

Finally, hydroxyectoine allowed long-term storage (half-life>185 days, included among the best results) of retroviral vectors, which are promising tools for gene therapy. This is a quite interesting effect, since a wider use of those vectors is currently hampered by loss of their infectivity during storage and transport (Cruz et al., 2006) (Table 2).

2.2.3. Cell protection capacity of ectoines

Ectoines do not only stabilize proteins and other macromolecules, but also are potent cell protectants (Table 2). Thus, ectoines added to the culture medium reversed the inhibition of *E. coli* growth caused by osmotic and temperature stresses (Malin and Lapidot, 1996). One of the mechanisms is the recovery of the glucose uptake activity (Nagata et al., 2002). When used with osmotically preconditioned bacteria, extracellular hydroxyectoine performs as well as extracellular trehalose for anhydrobiotic engineering of *E. coli* (Manzanera et al., 2004a). Louis et al. (1994) showed that ectoine and hydroxyectoine stabilized air-dried and freeze-dried *E. coli* cells during drying processes. In *P. putida*, hydroxyectoine was superior to trehalose as a drying excipient for storage (Manzanera et al., 2002), facilitating in this way a new method for drying and encapsulating the bacteria in plastic, demonstrating a potential application as a seed coating (Manzanera et al., 2004b). Ectoine also served as a specific osmoprotectant for the lactic acid bacterium *Tetragenococcus halophila*, which is used in soy sauce fermentation (Baliarda et al., 2003). Addition of ectoine improved cell growth and utilization of glucose, and protected the relative enzymes during ethanol fermentation in the Gram-negative ethanologenic bacterium *Zymomonas mobilis* (Zhang et al., 2008), which possesses advantages over *Saccharomyces cerevisiae*, since the former strain has a higher sugar–ethanol conversion (Jeffries, 2005) (Table 2).

Very recently, it has been reported that the supplementation of ectoine greatly improved the production of biodiesel by enzymatic conversion of triglycerides in cottonseed oil by using immobilized lipases, enhancing the reuse of the enzyme and significantly improving the methyl-ester concentrations in each recycling test (Wang and Zhang, 2010).

Nakayama et al. (2000a) transformed cultured tobacco cells with genes codifying for ectoine biosynthesis from *H. elongata*. Despite the low level of accumulation of this solute, the genetically engineered synthesis of ectoine resulted in an increased hyperosmotic tolerance. When those genes were later integrated in a stable manner into the tobacco genome by using an *Agrobacterium*-mediated gene delivery system, rates of ectoine accumulation were much higher, particularly in the roots. Ectoine protected the stomatal conductance and carboxylation activity, avoiding the depression of the photosynthetic rate by salinity (Moghaieb et al., 2006). The data revealed two ways in which ectoine enhanced salinity tolerance of tobacco plants. First, ectoine improved the maintenance of root function so that water was taken up consistently and supplied to shoots under saline conditions. Second, ectoine enhanced the nitrogen supply to leaves by increasing transpiration and by protecting Rubisco proteins from deleterious effects of salt, thereby improving the rate of photosynthesis (Moghaieb et al., 2006) (Table 2). This study opened the possibility to utilize the ectoine biosynthetic genes for the generation of transgenic crop plants that have an inbuilt enhanced tolerance to abiotic osmotic stresses, such as drought or salinity. Finally, *ectABC* operon genes from *M. halophilus* were transfected into tobacco plantlets via *Agrobacterium tumefaciens*. The transcripts were targeted to chloroplasts to become stabler. Ectoine

was detected in the transfected plants, although only with qualitative assays. As a result, increased salt tolerance from 100 to 300 mM at 25 °C, and higher stability of Rubisco activity were found in such plants (Rai et al., 2006).

2.2.4. Skin protection capacity of ectoines

The cumulative effect of external factors like radiation, wind, humidity and extreme temperatures leads to skin ageing (Rabe et al., 2006). It has been demonstrated that ectoine protects and stabilizes the membranes of pre-treated cells against the damaging effect of surfactants as well as the human skin to stress factors that would normally lead to skin dehydration (Bunger, 1999; Graf et al., 2008) (Table 2). Thus, ectoine functions as a more potent moisturizer than glycerol and features long-term moisturizing efficacy (Graf et al., 2008). Moreover, a long-term exposure to UVA could lead to photocarcinogenesis (Sander et al., 2004), immunosuppression (Wang et al., 2001), photodermatoses and photoageing (Fourtanier et al., 2006; Krutmann, 2000). Ectoines protect skin from the effects of UVA-induced cell damage in a number of different ways. One of the mechanisms of the UVA exposure damage is the formation of ceramide by a singlet oxygen-mediated mechanism. The exposure of primary human keratinocytes with UVA produces an increased ceramide level and, consequently, an intracellular signalling cascade is activated leading to the expression of the proinflammatory intercellular adhesion molecule-1. These negative effects are successfully prevented by ectoine due to its singlet oxygen-quenching properties (Buenger and Driller, 2004; Grether-Beck et al., 2005).

It has been described that in human dermal fibroblasts large-scale mutations of the mitochondrial DNA, the so-called 'common deletion', are time and dose-dependent of UVA irradiation (Berneburg et al., 1997, 1999; Buenger and Driller, 2004). This DNA damage is accompanied by a marked up-regulation of the matrix metalloproteinase-1 followed by skin wrinkle formation (Fligiel et al., 2003; Krutmann, 2000, 2003) but is prevented by ectoine. To restore cellular homeostasis, some mechanisms of cellular defense might be activated by inducing the expression of various genes, including the heat shock proteins (Hsps) (Giannessi et al., 2003). The expression of Hsp70 may be further induced in epidermal keratinocytes by heat treatment, providing a state of resistance against the deleterious effects of solar ultraviolet UV–B radiation (Souil et al., 2001). The Hsp70B9, a member of Hsp70 family, is strictly stress inducible and absent in unstressed cells (Leung et al., 1990; Tavaria et al., 1996). When keratinocyte cells were treated with ectoine and heat shocked, it was observed a marked increase of the constitutive form Hsp70 and a significant overexpression of Hsp70B9. The ability of ectoine to induce Hsps and downregulate proinflammatory signals results in a cytoprotective mechanism of keratinocytes (Buommino et al., 2005). Apart from the protection of skin from direct damage to keratinocytes, protection of Langerhans cells, key elements in the skin immune system, is provided too (Beyer et al., 2000; Buenger et al., 2001; Pfluecker et al., 2005; Tavaria et al., 1996) (Table 2).

Once the cells have been damaged to an extent that repair is no longer possible, cell death or apoptosis occurs to protect the body against the proliferation of such severely damaged and, hence, possibly mutating cells. These cells are formed in the human epidermis as a result of UV radiation and possess special characteristics — the so-called sunburn cells (SBCs) (Lizuka et al., 1988). There is distinct relationship between the formation rate of SBCs and the irradiation dosage administered. This relationship could, however, be displaced to a much higher UV dosage by pre-treating with ectoine (Buenger et al., 2001; Pfluecker et al., 2005).

Visible light also induces DNA damage as it produces DNA single-strand breaks (SSBs), sister chromatid exchanges (Sideris et al., 1981) and intracellular Reactive Oxygen Species (ROS) (Omata et al., 2006) which therefore exert an indirect genotoxic effect via oxidative DNA lesions (Pflaum et al., 1998). By using cells and keratinocytes, it was shown that DNA lesions induced by visible and UVA/visible light were reduced by ectoine, with maximal protective effects of 92.7% against visible light and 68.9% against UVA/visible light (Botta et al., 2008).

Because the activity of antioxidant enzymes and the levels of non-enzymatic antioxidants decline with age (Gonzalez-Ulloa and Flores, 1965; Tolmasoff et al., 1980), ectoine might prevent such oxidative damage in skin (Heinrich et al., 2007). In fact, its antiaging properties have been recently confirmed in a clinical trial comparing it with a high-quality skin care product (Heinrich et al., 2007).

2.3. Understanding the activity of ectoines

Compatible solutes are amphiphilic in nature and capable of "wetting" hydrophobic proteins. It has been suggested that compatible solutes reverse osmotic inhibition because they increase the total water content and, hence, the cytoplasmic volume of cells (Cayley et al., 1992). The structure-forming and breaking properties of compatible solutes indirectly influence the hydration shells and thus the activities of the proteins involved (Wiggins, 1990), as will be discussed below. There are different theories for explaining how the protective function of the compatible solutes in a low water environment works. Here we summarize the most important. The *preferential exclusion model* was proposed as a universal mechanism of protein stabilization by compatible solutes (Arakawa and Timasheff, 1985; Timasheff, 2002) (Fig. 4). This model proposes that due to unfavourable interaction with

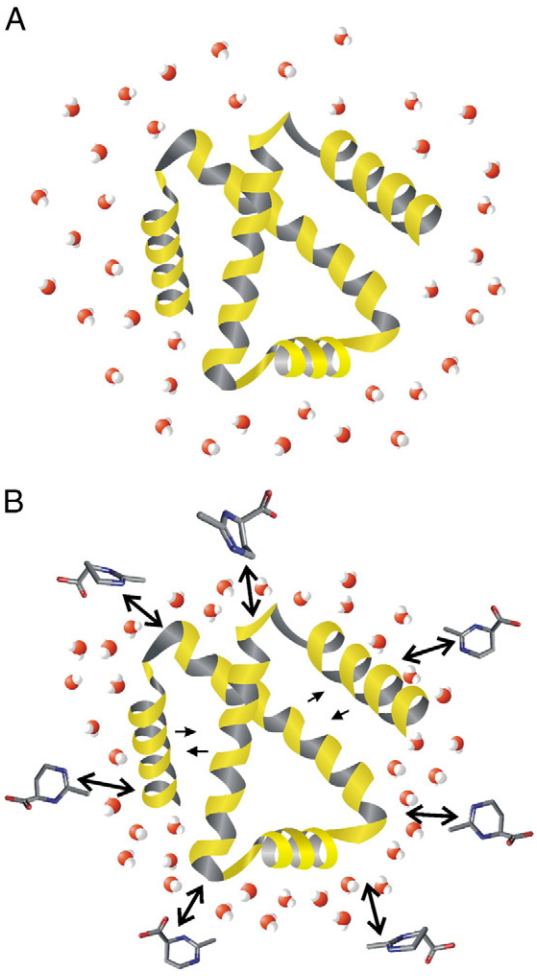

Fig. 4. Stabilization mechanism of compatible solutes. Based on the preferential exclusion model, the native conformation of a protein (A) is favoured by the presence of the compatible solutes molecules (B). A compact folded protein is generated by expelling the compatible solutes from the immediate hydration shell of the protein minimizing the volume from which the solute must be excluded. Small spheres represent water molecules and backbones represent compatible solutes (ectoine).

the surface of proteins, protective osmolytes are excluded from these, in contrast to denaturing solutes that are in contact with their surface. Thus, compatible solutes molecules are expelled from the immediate hydration shell of proteins and, consequently, unfolding needs additional energy and is disfavoured thermodynamically (Kurz, 2008; Lee and Timasheff, 1981). This, in turn, leads to preferential hydration of the protein because it is forced to occupy a smaller volume in order to minimize its exposed surface thus promoting its native conformation. Because compatible solutes do not interact directly with the protein surface, the catalytic activity remains unaffected (Galinski et al., 1997; Kolp et al., 2006).

According to this model, the so-called *'osmophobic' effect* explains how this effect can be universal (Bolen and Baskakov, 2001). Hydrogen bonds, Van der Waals, electrostatic and hydrophobic interactions are responsible for protein folding. Furthermore, it was argued that the unfavourable interaction of the solute with the peptide backbone provides the molecular basis for solute exclusion and, subsequently, the stabilizing effect (Liu and Bolen, 1995; Qu et al., 1998; Wang and Bolen, 1997). This osmophobic effect becomes relevant in highly concentrated solutions and in organisms that require high intracellular concentrations of osmolytes. In contrast to the hydrophobic effect which causes nonpolar amino acid residues to aggregate in the protein interior, the osmophobic effect influences the conformation of the peptide backbone. In fact, it has been suggested that the configuration of the protein backbone is the most important determinant of stabilization or denaturation (Burg and Ferraris, 2008). Any interactions of osmolytes with hydrophobic residues of the unfolded protein do not overcome the osmophobic effect, nor do they interfere with the hydrophobic effect (Roberts, 2005). Why are stabilizing solutes repelled by the protein backbone? Bennion and Daggett (2004) showed that methylamines alter water structure, causing greater organization through stronger hydrogen bonding among water molecules. Possibly, the peptide bond of proteins is less able to interact with (*i.e.*, be hydrated by) the organized water around methylamines than with bulk water (Yancey, 2005).

On the other hand, the *water replacement theory* (Clegg et al., 1982; Crowe et al., 1990) is based on the observation that many organisms are able to lose over 50% of cellular water, and to return to full activity after rehydration. Cellular structures can be protected by the accumulation of certain compatible solutes and their interactions with surfaces. Since, in this model, water is replaced by solutes, it seems to be the complete opposite of the *preferential interaction theory*. Replacement appears to be a very special situation at extremely low water activities, while the interaction model is valid for the more diluted range of solute concentrations. The importance of this model lies in the fact that the relative affinity of a solute towards water or protein may well be concentration dependent, especially when desiccation (*i.e.*, very low water activity) is involved (Kanias and Acker, 2006).

Manzanera et al. (2004a) showed that although hydroxyectoine performed well as an extracellular protectant, it was less effective than trehalose as an intracellular stabilizer. A possible explanation of this in the context of the water replacement hypothesis is that hydroxyectoine has only a single OH group per molecule, whereas trehalose has multiple free hydroxyl groups. *P. putida* was less desiccation tolerant in dried in trehalose than in hydroxyectoine, although no such difference is seen with *E. coli* (Manzanera et al., 2002). It was hypothesized that *P. putida* might contain desiccation-sensitive extracytoplasmic structures in the periplasm or outer face of the cytoplasmic membrane that are protected by hydroxyectoine, but are not accessible to trehalose as a result of porin selectivity.

According to Crowe et al. (1990), OH groups can partially replace water molecules. It was demonstrated that during extensive dehydration, there is extensive hydrogen bonding between OH groups of trehalose and polar residues of the proteins that preserve the conformational state of the dry proteins similar to that seen prior to dehydration. It was also demonstrated that trehalose directly interacts with the polar head groups of dry phosphatidylcholine, probably involving hydrogen bonding between OH groups on the trehalose and the phosphate, suggesting that trehalose can also have a stabilizing effect on lipid bilayers (Crowe et al., 1990). Lippert and Galinski (1992) speculated that free OH groups in hydroxyectoine and trehalose (but not in glycine betaine and ectoine) were required for effective stabilization of the enzymes assayed by formation of hydrogen bonds in macromolecules.

Ectoines have served as a model for the study of these interactions due to their biotechnological applications (Kurz, 2008; Lentzen and Schwarz, 2006). It is known that ectoine minimizes the denaturation that occurs upon the removal of water molecules by making the unfolding less favourable. In a study of proteases, it has been assumed that ectoine forces the unfold–fold equilibrium towards folded conformations, thereby preserving partial activity of these enzymes (Kolp et al., 2006). Water clusters around ectoine molecules remain stable for a long period of time, much longer than mixtures of water and glycerol that are disintegrated by the diffusion of water molecules out of the spheres (Graf et al., 2008). Thermodynamic aspects of protein stabilization by hydroxyectoine were studied using differential scanning calorimetry, with bovine ribonuclease A as a model protein. Calculation of the number of water molecules that additionally bind to unfolded RNase A resulted in surprisingly low number for this osmolyte (Knapp et al., 1999). Moreover, Yu and Nagaoka (2004) reported interesting results on molecular dynamic simulations performed for water–ectoine mixture models around chymotrypsin inhibitor 2 (CI2). According to their conclusions, ectoine maintains water at the surface by slowing down the water diffusion around a protein; the slowdown of water diffusion on the surface of the proteins should be the microscopic origin, which is connected to the kinetic and thermodynamic stabilization of the three dimensional structure of macromolecules.

By comparing the different interactions that were observed between CI2 and a smaller peptide, it was demonstrated that different parameters can influence in the preferential exclusion model (Yu et al., 2007). Thus, ectoine plays an indirect role in the alteration of the solvent properties and the modification of the stability of proteins (Göller and Galinski, 1999). However, it should be noted that the possible co-evolution of protein structures with cellular osmolyte compositions has so far received little attention.

3. Production processes for ectoines

The increasing commercial demand for ectoines has led to a number of efforts to improve their production from bacteria. In Table 1, microbial producers of ectoines, the different carbon sources used and production conditions are summarized.

Until middle nineties there was not any efficient, optimized method to produce ectoines in sufficient amounts to be commercially exploited, and it was either extracted from natural producers with low yields (Sauer and Galinski, 1998) or (and this was the main way) chemically synthesized (Koichi et al., 1991). To achieve a large-scale supply of ectoines in compliance with industrial demands, the production methods for ectoines have been optimized by using fed-batch and osmotic downshock ("bacterial milking") processes (Fig. 5). The reason why bacterial processes for the production of compatible solutes have been developed might be explained by the easier and higher specificity of product synthesis reached in comparison with the chemical synthesis, which generally consists of a series of reactions that lower the final product yield and may increase the number of separation steps and by-products generated. These by-products tend to be chemically similar to the aimed product, due to the low stereo-selectivity of chemical synthesis, which makes downstream purification processes even more difficult. Regio- and stereo-selectivity of enzymatic biotransformation enable for the specific production of the intended isomers. In addition, biotechnological processes tend to be far more environmentally friendly because the use of organic solvents

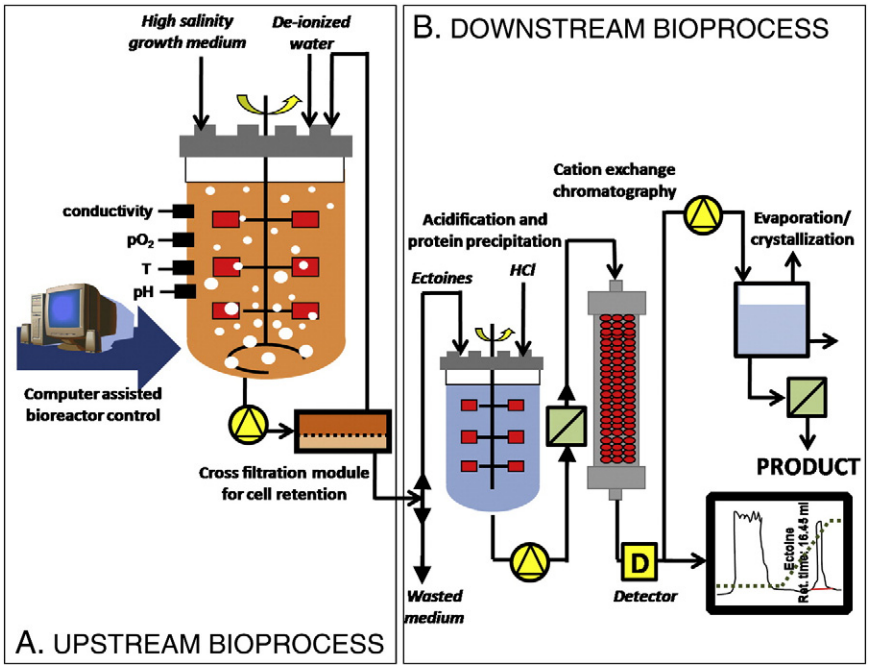

Fig. 5. General diagram of the industrial process for ectoine bioproduction.

and toxic chemicals is avoided. Bacterial methods, however, require high amounts of nutrients and finely tuned culture conditions such as pH, aeration, and nutrient feeding during the operation of fermentors. So far, mainly batch and fed-batch systems have been used for the production of ectoines (Tables 1, 3 and 4).

3.1. Ectoine

The "bacterial milking" is a fermentation technology procedure for large-scale internal metabolite extraction. These processes have been described in several microorganisms, such as *Escherichia coli* (Tsapis and Kepes, 1977), *Synechocystis* sp. (Reed et al., 1986) and some *Halorhodospira* species (Fischel and Oren, 1993), for the excretion of internal solutes. In the case of ectoine bioproduction, the bacterium *H. elongata* was chosen because of special characteristics that were not found in other species, namely a broad salt tolerance (being able to grow in media with a wide range of salt concentration) and, especially, their ability to respond to a hypoosmotic shock by rapid release of the intracellular ectoine pool and to rapidly resynthesize the excreted compatible solutes when transferred to a hyperosmotic medium (Fig. 5). *H. elongata* was subsequently applied as biofactory for the production of ectoine (Sauer and Galinski, 1998) (Table 3). In fact, the first process patented for ectoine production with such bacterium was held by the German firm Bitop AG (Galinski et al., 1994), using *H. elongata* ATCC 33173 as ectoine synthesizer.

Sauer and Galinski (1998) reported the aerobic culture of *H. elongata*, at 25 °C, in a medium with 15% w/v NaCl (2.57 M). The strategy consisted in setting-up a high cell-density fermentation (reaching up to 48 $g_{cdw}\,L^{-1}$) in a fed-batch reactor, supplying glucose as carbon source and ammonium chloride as nitrogen source (Table 3 and Fig. 5). Osmotic downshocks were carried out once a suitable optical density had been reached. Cross filtration was used to retain the cells, while replacing 80% of medium volume with distilled water. After one hour for the recovery of internal osmolarity of the cells, the same proportion of medium with excreted ectoines was withdrawn and replaced with the original salty medium, starting a new cycle of ectoine biosynthesis. The final maximum yield of ectoine reached was 155 mg g_{cdw}^{-1} (Table 3).

Microorganisms belonging to the genus *Brevibacterium* have also been regarded as feasible biofactories for the production of ectoines (Tables 1 and 3). Similarly to *H. elongata*, an osmotic downshock based method has been described using the *Brevibacterium* sp. JCM 6894 strain (Nagata et al., 2008). Unlike the method of Sauer and Galinski, the downshocks were made by centrifuging the biomass and resuspending the cell pellets on distilled water (Table 3). However, ectoine yield and productivity were lower than those achieved by a formerly described process on *Brevibacterium epidermis*, which did not use periodic downshocks, but a final extraction process using distilled water and ethanol (Onraedt et al., 2005) and salt concentration as low as 1 M. This is an important point, since one of the main problems of this kind of processes is the drawbacks that working with high salt concentrations involve, mainly the corrosion of the equipment and the reduction in growth rate and maximum density, eventually leading to lower ectoine production (Table 3).

Another way to dodge the drawbacks of high salinity media in industrial processes of ectoines production might be the use of strains which could both naturally synthesize this compound and thrive under relatively low salinity conditions. Following this strategy, Zhang et al. used an ectoine-excreting strain of *Halomonas salina* grown in 0.5 M NaCl batch fermentors and with monosodic glutamate as a carbon and nitrogen source. They also carried out parallel cultures of *H. elongata* DSM 2581^T, a non-ectoine-excreting strain to use them for comparison. This study also indicated the potential application of free or immobilized cells for continuous culture to produce ectoine (Zhang et al., 2009).

In most microorganisms, the osmoprotection response involves the formation of more than one compatible solute. In Tables 1 and 3, it is depicted that parallel to the production of ectoines, other compounds can be also obtained, including amino acids such as glutamate, proline and alanine, and others. It is the case of the halophile *Halomonas boliviensis*, for which a co-production process of ectoine and the biopolyester poly(3-hydroxybutyrate) (PHB) has been described. Thus, by using fed-batch fermentation and harvesting each product sequentially, it was obtained a slightly lower to similar yield of ectoine as those reached in previously described processes where ectoine is the sole target compound. Moreover, hydroxyectoine was also obtained (50 mg L^{-1}) at 15% (w/v) NaCl (Guzman et al.,

Table 3
Reactor systems used for ectoines production.

Microorganism	Product	By-products	Production yield (mg g_{cdw}^{-1})	Productivity (g L^{-1} day^{-1})	Maximum biomass (g L^{-1})	Reactor system	Product extraction	Carbon source	NaCl	Ectoines excretion upon osmotic downshock	References
Actinobacteria											
Brevibacterium epidermis DSM 20659	Ectoine		160	2	50	Fed-batch	Water downshock Ethanol extraction	Sodium glutamate yeast extract	1 M	50%	Onraedt et al. (2005)
Brevibacterium sp. JCM 6894	Ectoine		150	0.34	NR	Batch	Water downshock	Polypeptone yeast extract	2 M	50%	Nagata et al. (2008)
Firmicutes											
Marinococcus sp. M52	Hydroxyectoine	Glutamate	135	1.21	56	Fed-batch–Batch [10]	Methanol extraction	Glucose and fish peptone	1.71 M	0%	Frings et al. (1995)
	Hydroxyectoine		70–80	0.8–0.9	45	Batch–Fed-batch [10]	Thermal permeabilization[11]	Glucose fish peptone	1.54 M	0%[15]	Schiraldi et al. (2006)
Proteobacteria											
Chromohalobacter salexigens DSM 3043	Ectoine Hydroxyectoine		540 (Ect) 400 (OH-Ect)	32.5 (Ect) 17.5 (OH-Ect)	61	Continuous with cell retention	Downshocks	Glucose	1.84 M	95%	Fallet et al. (2010)
Escherichia coli DH5α[1]	Ectoine		270	0.96	22	Batch	Excreted by the cells	Glucose	0 M	–	Schubert et al. (2007)
Halomonas boliviensis DSM 15516	Ectoine PHB	Hydroxyectoine	170	3.4	33.5	Two step fed-batch	Thermal permeabilization [11]	Glucose and sodium glutamate[12]	2.13 M[14]	95%	Guzman et al. (2009)
	Ectoine Hydroxyectoine	PHB[2]	154 (Ect) 36 (OH-Ect)	9.1 (Ect) 2.0 (OH-Ect)[8]	62.4[9]	Two step fed-batch	Downshocks	Glucose and sodium glutamate[13]	2.55 M[14]	75%	Van-Thuoc et al. (2010)
Halomonas elongata DSM 142	Ectoine	Hydroxyectoine	155[3]	5.3[4] 1.3[5]	48	Batch–Fed-batch	Downshocks	Glucose	2.57 M	90%	Sauer and Galinski (1998)
Halomonas salina DSM 5928	Ectoine		358[6], 220[7]	7.9	19.4	Batch	Excreted	Sodium glutamate	0.5 M	–	Zhang et al. (2009)

NR = not reported.
1. Carrying plasmid pASK with *ectABC* operon from *C. salexigens* DSM 3043.
2. PHB by-production increased with downshock extraction cycles.
3. In mg g_{cdw}^{-1} $cycle^{-1}$.
4. Calculated for the whole process until the first extraction cycle.
5. Calculated for the whole process until the 9th cycle.
6. Total ectoine content.
7. Excreted ectoine.
8. Maximum productivity of hydroxyectoine was 5.7 g L^{-1} day^{-1}, reached on 3.15 M of NaCl at 2nd fed-batch.
9. Maximum cell density was 78.6 g L^{-1}, reached on 2.13 M of NaCl at 2nd fed-batch.
10. With medium exchange once, during fed-batch.
11. Optimum permeabilization temperature was 55 °C.
12. From early 2nd fed-batch, glutamate was not fed, to enhance PHB production.
13. Glutamate fed only on the 1st fed-batch.
14. Given salt concentration is from 2nd fed-batch; at 1st fed-batch it was 0.77 M.
15. Downshock at room temperature. 100% of extraction upon thermal permeabilization.

Table 4
Heterologous producer characteristics.

Host	Origin	Genes	Heterologous genes location	Product yield	Salt range increase	References
Escherichia coli	*Marinococcus halophilus*	*ectA*, *ectB*, *ectC*, *orfA*[1]	Plasmid	1[2]	From 0.51 to 0.86 M	Louis and Galinski (1997)
Escherichia coli	*Chromohalobacter salexigens* DSM 3043	*ectA*, *ectB*, *ectC*	Plasmid	ND	ND	Canovas et al. (1998)
Bacillus subtilis	*Bacillus pasteurii*	*ectA*, *ectB*, *ectC*	Genome	ND	ND	Kuhlmann and Bremer (2002)
E. coli DH5α	*Halomonas* sp. BIS-1	*ectA*, *ectB*, *ectC*	Plasmid	0.003[2]	ND	He et al. (2006)
E. coli DH5α	*Chromohalobacter salexigens*	*ectA*, *ectB*, *ectC*	Plasmid	1.9[3]	ND	Schubert et al. (2007)
E. coli DH5α	*Marinococcus halophilus*	*ectA*, *ectB*, *ectC*	Plasmid	0.4[2]	From 0.51 to 0.86 M	Bestvater et al. (2008)
Escherichia coli	*Bacillus halodurans*	*ectA*, *ectB*, *ectC*	Plasmid	6[3]	ND	Rajan et al. (2008)
Chromohalobacter salexigens DSM 3043	*Streptomyces crysomallum*	*thpD*	Plasmid	100[4]	ND[5]	Prabhu et al. (2004)
Nicotiana tabacum	*Marinococcus halophilus*	*ectA*, *ectB*, *ectC*	Plasmid	ND	From 0.1 to 0.3 M at 25 °C	Rai et al. (2006)

1. *orfA* is not related to metabolism of ectoines.
2. mmol of product/gram of cell dry weight.
3. mg/L of product (biomass not tested).
4. Percentage of ectoine conversion to hydroxyectoine, absolute yield not tested.
5. Host organism is already halotolerant.

2009). More recently, two cultivation steps were used for production of biomass and ectoine by the same bacterium, respectively. The optimization of some nutrient parameters in each step was investigated by using response surface methodology. Ectoine concentration of 6 g L^{-1} was obtained, and the overall ectoine productivity was 9.1 g L^{-1} day^{-1}, being among the highest reported so far (Table 3) (Van-Thuoc et al., 2010).

Very recently, Fallet et al. (2010) used an integrated process consisting of two continuous bioreactors connected in series. This cascade outperformed hitherto reported processes for ectoine production in terms of titers and productivity (see Table 3) and exemplifies the potential of process optimization by continuous cultivation with integrated downstream processing modules.

3.2. 5-Hydroxyectoine

The hydroxylated derivative of ectoine, hydroxyectoine, has also been a target of economic interest, due to its specific biological features that make it different from other compatible solutes, such as its applicability to improve the thermoresistance of proteins and cells, and a greater stabilization capacity than ectoine (see previous sections). Hence, several processes have been described to exploit it efficiently. The drawbacks of the chemical synthesis of ectoine are even more relevant to hydroxyectoine, because that the hydroxyl group adds another chiral center to the molecule, what makes the biotechnological alternative even more interesting. *Marinococcus* M52 (Tables 1 and 3) is so far the most exploited microorganism to this purpose, yielding high amounts of hydroxyectoine from ectoine during the stationary phase of culture (Frings et al., 1995). A high cell-density method applying exponential feeding and downshock extraction for hydroxyectoine production by this strain was developed, reaching 134.8 mg g_{cdw}^{-1}. These results were further improved by Schiraldi et al. (2006), who optimized dissolved oxygen concentration and growth media composition to obtain a maximal hydroxyectoine yield. Thus, dissolved oxygen content higher than 10% during cultivation led to more rapid accumulation of hydroxyectoine than of ectoine (with hydroxyectoine up to 1.6 g L^{-1}). In addition, a novel extraction method based on osmotic downshock coupled with thermal permeabilization to recover the desired products from the biomass was also implemented, improving the product extraction step and, in doing this, also the productivity. However, the permeabilization step does not allow the application of ectoine synthesis/extraction cycles by reutilizing the cells, which is therefore a limitation for bioprocess optimization. Finally, by optimizing a two-step fed-batch cultivation of the halophilic bacterium *H. boliviensis*, Van-Thuoc et al. (2010) achieved a hydroxyectoine productivity of 2.0 g L^{-1}, which is 2 to 5 times higher than that achieved by *Marinococcus* M52. This hydroxyectoine productivity ranges among the highest reported so far (Table 3).

3.3. Heterologous ectoine producers

In parallel to all described above, many studies involving heterologous expression of ectoine synthesis pathways both in prokaryotic and eukaryotic organisms have been carried out (Table 4). Two are the main objectives that have boosted them, on the one hand, the benefits of transferring abiotic stress resistance to organisms that lack it naturally, in order to improve some interesting biological properties, and on the other hand, the advantages of getting rid of the drawbacks that arise from the use of high salt media in industrial ectoine production processes. In the latter case, prokaryotes are almost uniquely used as host organism, since they are much more suitable for the systematic transfer to high-scale bioprocesses and because of the availability of techniques for their genetic modification. *E. coli* is by far the most exploited host, as it is a model organism for biotechnological genetic engineering applications. In the last two decades, *E. coli* has been used as host for the heterologous expression of the ectoine synthesis gene cluster (which often occurs in nature as an operon), *ectA*, *ectB* and *ectC* from different halophiles (Fig. 3). The most used sources have been *M. halophilus* and *C. salexigens*, among others (Rajan et al., 2008) (Table 4).

M. halophilus ectoine synthesis genes are homologous to those of *H. elongata* and *C. salexigens* and have the same organization in a cluster (*ectABC*). They were first cloned and expressed in an *E. coli* strain, from a gene library (Louis and Galinski, 1997). *E. coli* is able to grow at salt concentrations of up to 0.5 M NaCl in minimal medium because of its intrinsic ability to adjust its cytoplasmic potassium glutamate and trehalose pools. At 0.8 M NaCl, more efficient compatible solutes, such as betaine or ectoine, are needed for growth, whereas on salt concentrations higher than 1 M, even externally supplied compatible solutes cannot make growth possible (Le Rudulier and Bouillard, 1983). Clones from the gene library with increased osmotolerance were selected and heterologous genes therein cloned were identified as *ectA*, *ectB* and *ectC*, thus showing, for the first time, how a non-halophilic microorganism can express functionally genes of a salt stress pathway. Clones carrying the *ectABC* genes synthesized up to 1 mmol g_{cdw}^{-1} at 0.85 M NaCl. Further studies on heterologous expression of *M. halophilus* genes in *E. coli* were subsequently carried out, focusing on the regulatory mechanisms involved in the synthesis of both ectoine and aspartic derived amino acids that did not allow thorough expression of *ectABC* genes in *E. coli* (Bestvater et al., 2008). Feedback inhibition and/or repression by L-lysine, L-threonine and L-methionine, which normally operates in *E. coli*, showed to be the main mechanism hampering the transformed strain to reach intracellularly synthesized ectoine levels as high as those reached by uptake from the medium in the wild type strain. This fact was

demonstrated by providing transformed *E. coli* with an allosterically deregulated aspartate kinase from *Corynebacterium glutamicum*. Moreover, as first described Louis and Galinski (1997), expression of *ectABC* genes promoted growth of *E. coli* at salinities higher than 0.5 M (Bestvater et al., 2008). However, none of these studies achieved higher accumulation or excretion of ectoines from natural producers such as the *M. halophilus* or *Brevibacterium* species.

On the other hand, the transformation of *E. coli* cells with ectoine biosynthesis genes from *H. elongata* (He et al., 2006) or *C. salexigens* (Canovas et al., 1998; Schubert et al., 2007) failed to promote growth of host cells on high salinity media, but reached high intracellular yields of ectoine. The ectoine genes of *C. salexigens* were introduced into an *E. coli* strain using an expression vector under the control of a *tet* promoter. The transgenic *E. coli* synthesized 6 g L^{-1} ectoine with a space-time yield of 40 mg L^{-1} h^{-1}, with the vast majority of the ectoine being excreted (Schubert et al., 2007).

The use of other species as expression host has been approached as was the case of *B. subtilis* and *C. salexigens* DSM 3043 (Kuhlmann and Bremer, 2002; Prabhu et al., 2004). Ectoine synthesis genes from one of the main producer species, namely *B. pasteurii*, were cloned in *B. subtilis* cells, which synthesize proline as main compatible solute, and their heterologous expression led to the production of ectoine. Another study was focused on the improvement of the hydroxyectoine"milking" bioproduction process. By cloning and expressing *thpD*, the ectoine hydroxylase encoding gene of *Streptomyces crysomallus*, in *C. salexigens*, a 100% of conversion of the ectoine naturally produced to hydroxyectoine was achieved (Prabhu et al., 2004). This allowed the production of hydroxyectoine at a lower salt concentration (1.7 M NaCl), although the absolute yield on hydroxyectoine was not reported. It should be underlined that this is the only case of a heterologous hydroxyectoine producing microorganism so far described in the literature. In fact, the non-engineered *Marinococcus* M52 strain continues to be the preferred choice for the industrial scale production of hydroxyectoine.

4. Production systems

The first step to be taken before the setting-up of an industrial bioproduction is the choice of the production system, in this case the ectoine/hydroxyectoine producer microorganism. Such system has to accomplish some basic requirements that have to be analyzed prior to the design of the process. Growth temperature and pH must not be extreme, as it would impose severe restrictions when scaling-up, probably affecting the equipment used. The producer microorganism could be a natural producer of ectoines or could be an engineered strain of a non-halophile. Aerobic microorganisms are preferred, since respiration yields much higher biomass. Optimal salt concentration for maximum ectoine synthesis must also be taken into account, because of the drawbacks imposed by high salinity media, as previously explained.

Since ectoines are osmoprotectants, they usually accumulate intracellularly in order to offset external osmolarity, which implies the need of a proper extraction process. The applicability of the "milking" method generally used for the extraction of ectoines is also conditioned by the production system chosen. Gram-negative bacteria are much more sensitive to external salinity changes, and the survival rate depends on the way in which the downshock is applied, especially on the changes in the salinity range. On the other hand, Gram-positive bacteria withstand osmotic changes much better than the Gram-negative because of their rigid cell wall, but do not excrete intracellularly accumulated compatible solutes upon downshock treatment, as in the case of *Marinococcus* sp. M52 (Frings et al., 1995). Consequently, medium exchange and osmotic downshock are normally used in Gram-negatives to extrude ectoines, whereas in Gram-positives it is required to desalt the medium prior to the extraction of ectoines by other methods.

This "milking" process imposes some restrictions in process performance and development, and complicates downstream processing. Consequently, the use of microorganisms that are able to excrete ectoines without requiring external osmolarity changes has been desired. For this aim, it is most likely essential to use an engineered non-halophilic microorganism. A successful example of this was *E. coli* carrying the *ectABC* cluster from *C. salexigens* (Schubert et al., 2007).

As stated above, there are two main types of production systems suitable to approach ectoine bioproduction: non-halophilic microorganisms bearing heterologous ectoine synthesis genes, and natural (halophilic) producers, cultured in optimal conditions for ectoine accumulation. Depending on which of the two production systems is chosen, different specific advantages or drawbacks may arise, which condition the subsequent setting-up of the reactor system. Many examples of both natural and engineered ectoine producers have been given throughout previous sections. In most cases, the natural producers have been shown to yield higher levels of ectoine accumulation, partly because of the absence of the metabolic bottlenecks imposed by the central metabolism of the host microorganism to the optimal performance of the heterologous production pathway, as demonstrated in the case of *E. coli* (Bestvater et al., 2008). In addition, the use of heterologous organisms involves certain drawbacks derived from the use of plasmids as gene vectors, which, so far, is the most common choice when generating ectoine synthesizing heterologous microorganisms. The development of proper culture media and feeding strategies, allowing optimal ectoine production and recovery while supporting high cell-density cultivation is a major drawback for many halophilic bacteria.

Another feasible strategy, which is halfway between these two, is the improvement of halophilic microorganisms by strain engineering. *H. elongata* cells bearing the *thpD* gene from *Streptomyces crysomallum* for hydroxyectoine synthesis improvement is an example of this (Prabhu et al., 2004). However, most scalable processes for ectoine production described so far, use natural producers and "milking" techniques. Thus, further efforts on strain improvement and the generation of optimal halophilic and non-halophilic recombinant producer strains are still needed in order to increase productivity and avoid the drawbacks from classical bioprocesses.

5. Reactor systems

According to the specific features of the production system chosen, a suitable reactor configuration must be chosen to allow the proper performance of the production process. Since the main role of ectoines is to offset external osmolarity by accumulating intracellularly, cells have to synthesize them constantly during growth, although they are specially accumulated once the stationary phase of culture is reached. Therefore, for high production of ectoines it is important to achieve high cell density in the fermentor. High cell-density cultivations (HCDC) are a type of fermentation that aim to achieve the highest possible biomass density. These procedures have been applied for a long time for bioproduction of antibiotics, recombinant proteins, metabolites, polymers, etc., mainly in yeasts and mesophiles (Bernal et al., 2007; Canovas et al., 2003; Obon et al., 1997; Riesenberg and Guthke, 1999; Shiloach and Fass, 2005) and, more recently (from early nineties), also in some types of extremophiles, among them several halophiles (Schiraldi and De Rosa, 2002). The maximum cell densities reached in optimized HCDC vary widely depending on the nature of the cultured microorganism. Traditional HCDC of mesophiles and yeasts used to reach 100–250 g L^{-1} (Riesenberg and Guthke, 1999), whereas in halophiles those values are usually lower. In the specific case of ectoine producers, it is in the range of 40–60 g L^{-1} (Table 3) (Fallet et al., 2010; Frings et al., 1995; Guzman et al., 2009; Onraedt et al., 2005; Sauer and Galinski, 1998; Schiraldi et al., 2006; Van-Thuoc et al., 2010).

It is well-known that fed-batch reactors allow reaching higher maximal biomass levels than batch reactors, since growth limitation because of the exhaustion of some essential component of the medium is avoided by periodically feeding with either fresh medium or selected nutrient(s). Moreover, biomass level can be further improved in a fed-

batch reactor, applying cross-flow filtration to replace the growth medium by fresh broth, therefore getting rid of excreted metabolites, such as certain organic acids, that would inhibit cell growth. Maximum cell density achievable with a given microorganism depends strongly on the culture conditions, namely pH, aeration, medium composition and temperature (Riesenberg and Guthke, 1999; Shiloach and Fass, 2005). For most processes described so far in the literature several of these parameters had to be optimized to increase maximum biomass levels. As discussed above, optimal temperature and pH rarely reach extreme values, and, therefore, they are normally easy to control and scale-up. In the case of aerobic microorganisms, higher oxygen supply is demanded at high cell densities, and high stirring and aeration rates have to be applied. However, at the final stages of fed-batch cultivations, when the highest cell densities are reached, it is more difficult to keep such aeration (Riesenberg and Guthke, 1999). This fact can be critical in some cases, especially those in which aeration directly affects the rate of product synthesis. This was the case of hydroxyectoine production by *Marinococcus* sp. M52 (Schiraldi et al., 2006). At a given pO_2 value, hydroxyectoine/ectoine synthesis ratio was constant during the exponential phase, and increased in stationary phase. The increase in the dissolved oxygen (pO_2) in the culture broth allowed to further increase the hydroxyectoine synthesis ratio in the exponential phase of culture.

HCDC performance is also affected by the composition of the culture medium. At high concentration, carbon and nitrogen sources can inhibit growth, and other nutrients and salts may precipitate. For this reason, HCD fed-batch cultivations use different strategies of semicontinuous fresh medium supply during the process, to keep concentration of nutrients in a suitable range of concentrations (Frings et al., 1995; Schiraldi et al., 2006). In many cases, medium composition also has to be optimized for maximum ectoine production. Glucose as carbon and energy source and ammonium salts for nitrogen source are normally used (Table 3). Fish peptone/glucose proportion has been adjusted for optimal hydroxyectoine synthesis by *Marinococcus* sp. M52 cultivations, and sodium glutamate has been used as both carbon and nitrogen source for *B. epidermis* (Table 3) (Onraedt et al., 2005).

In some instances, two or more reactors have been coupled in series in order to separately optimize the cellular growth and bioproduction processes. A useful strategy is to apply a fed-batch process for obtaining high cell mass at a first stage and, following a filtration step, transferring the cells to a second fed-batch reactor with fresh medium where bioproduct synthesis/excretion occurs. This strategy is especially useful when the conditions for optimal growth are different from those for optimal bioproduct synthesis. It has been applied for ectoine production (Van-Thuoc et al., 2009, 2010), and for simultaneous obtention of ectoine and poly(3-hydroxybutyrate) (Guzman et al., 2009).

Although fed-batch cultures are generally chosen for HCDC, there are other options that can provide further advantages. Continuous reactors permit a finely tuned control of variables that cannot be performed with batch type reactors, and allow reaching a metabolic/physiologic stationary state, which favours the reproducibility. On standard continuous reactors, maximum biomass is lower than in batch type reactors, because of continuous loss of cells at the reactor outlet. However, HCD can be achieved by coupling a cell retention system, such as cross-flow filtration, or by cellular immobilization (Canovas et al., 2003; Obon et al., 1999; Riesenberg and Guthke, 1999; Shiloach and Fass, 2005). This has been recently applied for ectoine production by *C. salexigens* DSM 3043 in a system of two coupled continuous reactors, the first aimed obtaining high cell mass and, in the second one, the high cell-density broth from the former was downshocked with hypoosmotic medium to release the synthesized ectoines (Fallet et al., 2010). To date, this is the only case of an ectoine production process with continuous reactors. The excellent productivity reported by these authors encourage further investigations in the development of continuous cultivation systems for the production of compatible solutes.

The main advantage of the bacterial "milking" process is that it allows extracting the desired product repeatedly, without significant loss of cell viability. However, as previously explained, it is only applicable to Gram-negative microorganisms. In fact, hypoosmotic downshock is widely used as extraction procedure in many ectoine production processes (Fallet et al., 2010; Nagata et al., 2008; Sauer and Galinski, 1998; Van-Thuoc et al., 2010). In the case of Gram-positive bacteria, the culture broth is exchanged with demineralized water, followed by extraction with organic solvents (generally, methanol or ethanol) for thorough separation of ectoines (Frings et al., 1995; Onraedt et al., 2005). To avoid the use of organic solvents for extraction, a method of thermal permeabilization has been described, solely using distilled water for medium exchange (Schiraldi et al., 2006). The main disadvantage of these extraction/permeabilization methods is that cells cannot be recycled back to the reactor, which lowers final process productivity. In addition, the type of product recovery method has a large impact on the downstream processing operations. While downshocks performed with distilled water permit a better removal of salts prior to extraction with organic solvents or thermal permeabilization, very often, downshock for milking processes must be performed in the presence of a minimal salt concentration to keep the cells viable. Therefore, product thus obtained has to be subsequently desalted during purification (Fallet et al., 2010; Sauer and Galinski, 1998; Van-Thuoc et al., 2010). In the industrial processes, separation and purification operations are coupled to the "milking"/extraction stage. If the milking process has been used for extraction, desalting and concentration is performed by chromatographic techniques, namely cation exchange or ion-retarding resins (Sauer and Galinski, 1998). On the other hand, ectoine extraction with organic solvents carries over many more contaminants, such as salts and cellular proteins and metabolites, and several additional purification steps (centrifugation/filtration) are needed (Frings et al., 1995; Onraedt et al., 2005), which imposes novel constraints for process development.

6. Perspectives

In general, current methodologies for high scale production of ectoines involve fed-batch high density cultures and "bacterial milking" for extraction (Table 3). These bioprocesses are affected by a number of drawbacks which remain to be tackled.

Since the high salinity needed in most of the processes described so far (up to 2.5 M, Table 1) may affect the equipment used for cultivation and the subsequent downstream processes, the identification of novel natural ectoine-producing strains or the development of new engineered biocatalysts able to produce ectoines at low or moderate salt concentrations should be a major objective to be pursued. Additionally, the strains should be able to grow to high cell densities and the processes should be adaptable to the high scales needed for industrial production. There is room for the improvement of downstream operations. Recently, the development of an ectoine extraction procedure involving cellular permeabilization has underlined the advantages of more environmentally friendly techniques (Schiraldi et al., 2006). Moreover, the improvement of bacterial milking or even the development of ectoine-excreting strains can also be envisaged (Zhang et al., 2009).

The next generation technologies may be also focused on ectoine-producing strains that may use inexpensive stocks of carbon; example could be CO_2 (phototrophs)- or CH_4(methanotrophs)-based technology. Several phototrophic and methanotrophic ectoine producers were reported. Exploring technological potential of theses cultures is very interesting; as such technologies could be beneficial for environment if they are linked to CO_2 or CH_4 mitigation.

As stated before, further efforts are needed in order to improve high scale microbial culture strategies, particularly for extremophile microorganisms. A higher efficiency in biomass production will depend on the ability to redirect metabolic fluxes to optimize growth and to adapt current culture strategies to meet microbial needs by means of the control of parameters such as stirring, pH, salinity and aeration. A better description of central metabolism of producer strains is needed, with a special focus on the ability to use the carbon source for the generation of energy and metabolic building blocks and, at the same time, a balanced generation of ectoines. In order to improve metabolic efficiency, futile cycles and metabolic overflow mechanisms should be described, understood and minimized. Knowledge on the regulatory mechanisms involved in the coordinated control of ectoine and aspartate-derived amino acids should be gained as well, so as to identify metabolic bottlenecks to be targeted by genetic engineering. This will involve a thorough biochemical characterization of the enzymes involved and their regulation by end products.

Finally, a Systems Biology approach for the understanding of the ectoines production process, describing the connection of biosynthetic pathways with central metabolism is still needed. Metabolic models describing the activity of the strains will be the basis for the identification of optimization strategies. A combined model of reactor and metabolism, linking the macrokinetics of the reactor with the cellular microkinetics, should show control points at macroscopic (reactor operation) and microscopic (molecular) levels where conversion and productivity could be increased.

7. Conclusions

In this review, we have discussed current uses and importance of ectoines, from the protection and stabilization of proteins and nucleic acids to their role as a therapeutic for certain diseases by preventing whole cell damage and loss of viability. The high number of applications so far identified, together with the several novel ones which are envisaged in the next years will surely result in a growing world wide commercial demand for ectoines. In the last years, this fact has led to a multiplication of efforts to improve their production from bacteria.

Current methodologies for high scale ectoine production stem from initial fundamental studies of their role in microbial osmoprotection. Bioprocess engineers have developed scalable methodologies for industrial cultivation of halophilic microorganisms and for the recovery of these compatible solutes. So far, molecular engineering efforts for the generation of recombinant mesophilic producers have not been very successful. In order to develop novel processes with improved performance, new strategies have to be designed and Metabolic Engineering and Systems Biology are highly valuable tools to rationally approach the development of engineered microorganisms. Therefore, a closer link between fundamental and applied research is foreseen.

Acknowledgements

This work has been supported by grants from MICINN project BIO2008-04500-C02-01 and BIO2O08-04117, and Junta de Andalucia PO8-CVI-03724. J.M. Pastor, M. Salvador and M. Reina-Bueno were recipients of a fellowship from the Spanish Ministerio de Ciencia e Innovación (MICINN). V. Bernal holds a contract with the University of Murcia funded by Fundación Séneca (Murcia, Spain).

References

Aguzzi A, Polymenidou M. Mammalian prion biology: one century of evolving concepts. Cell 2004;116:313–27.

Andersson MM, Breccia JD, Hatti-Kaul R. Stabilizing effect of chemical additives against oxidation of lactate dehydrogenase. Biotechnol Appl Biochem 2000;32:145–53.

Arahal DR, Garcia MT, Vargas C, Canovas D, Nieto JJ, Ventosa A. *Chromohalobacter salexigens* sp. nov., a moderately halophilic species that includes *Halomonas elongata* DSM 3043 and ATCC 33174. Int J Syst Evol Microbiol 2001;51:1457–62.

Arakawa T, Timasheff SN. The stabilization of proteins by osmolytes. Biophys J 1985;47: 411–4.

Argandoña M, Nieto JJ, Iglesias-Guerra F, Calderón MI, García-Estepa R, Vargas C. Interplay between iron homeostasis and the osmotic stress response in the halophilic bacterium *Chromohalobacter salexigens*. Appl Environ Microbiol 2010;76:3575–89.

Arora A, Ha C, Park CB. Inhibition of insulin amyloid formation by small stress molecules. FEBS Lett 2004;564:121–5.

Aston JE, Peyton BM. Response of *Halomonas campisalis* to saline stress: changes in growth kinetics, compatible solute production and membrane phospholipid fatty acid composition. FEMS Microbiol Lett 2007;274:196-3.

Azam TA, Ishihama A. Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. J Biol Chem 1999;274:33105–13.

Baliarda A, Robert H, Jebbar M, Blanco C, Deschamps A, Le Marrec C. Potential osmoprotectants for the lactic acid bacteria *Pediococcus pentosaceus* and *Tetragenococcus halophila*. Int J Food Microbiol 2003;84:13–20.

Banciu HL, Sorokin DY, Tourova TP, Galinski EA, Muntyan MS, Kuenen JG, et al. Influence of salts and pH on growth and activity of a novel facultatively alkaliphilic, extremely salt-tolerant, obligately chemolithoautotrophic sufur-oxidizing Gammaproteobacterium *Thioalkalibacter halophilus* gen. nov., sp. nov. from South-Western Siberian soda lakes. Extremophiles 2008;12:391-04.

Barth S, Huhn M, Matthey B, Klimka A, Galinski EA, Engert A. Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. Appl Environ Microbiol 2000;66:1572–9.

Bennion BJ, Daggett V. Counteraction of urea-induced protein denaturation by trimethylamine N-oxide: a chemical chaperone at atomic resolution. Proc Natl Acad Sci USA 2004;101:6433–8.

Bernal V, Sevilla A, Canovas M, Iborra JL. Production of L-carnitine by secondary metabolism of bacteria. Microb Cell Fact 2007;6:31.

Bernard T, Jebbar M, Rassouli Y, Himdikabbab S, Hamelin J, Blanco C. Ectoine accumulation and osmotic regulation in *Brevibacterium linens*. J Gen Microbiol 1993;139:129–36.

Berneburg M, Gattermann N, Stege H, Grewe M, Vogelsang K, Ruzicka T, et al. Chronically ultraviolet-exposed human skin shows a higher mutation frequency of mitochondrial DNA as compared to unexposed skin and the hematopoietic system. Photochem Photobiol 1997;66:271–5.

Berneburg M, Grether-Beck S, Kurten V, Ruzicka T, Briviba K, Sies H, et al. Singlet oxygen mediates the UVA-induced generation of the photoaging-associated mitochondrial common deletion. J Biol Chem 1999;274:15345–9.

Bersch S, Vangala M, Schwarz T, Kaufmann M. Protection of antibodies against proteolytic degradation by compatible solutes; 2000. Lissabon.

Bestvater T, Galinski EA. Investigation into a stress-inducible promoter region from *Marinococcus halophilus* using green fluorescent protein. Extremophiles 2002;6:15–20.

Bestvater T, Louis P, Galinski E. Heterologous ectoine production in *Escherichia coli*: bypassing the metabolic bottle-neck. Saline Systems 2008;4:12.

Beyer N, Driller H, Bunger J. Ectoine, an innovative, multifunctional, active substance for the cosmetic industry. SOWF J 2000;126:26–9.

Bolen DW, Baskakov IV. The osmophobic effect: natural selection of a thermodynamic force in protein folding. J Mol Biol 2001;310:955–63.

Botta C, Di Giorgio C, Sabatier AS, De Meo M. Genotoxicity of visible light (400–800 nm) and photoprotection assessment of ectoin, L-ergothioneine and mannitol and four sunscreens. J Photochem Photobiol 2008;91:24–34.

Bremer E, Krämer R. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. In: Storz G, Hengge-Aronis R, editors. Bacterial stress responses. Washington, D.C.: ASM Press; 2000. p. 77–97.

Bringardner BD, Baran CP, Eubank TD, Marsh CB. The role of inflammation in the pathogenesis of idiopathic pulmonary fibrosis. Antioxid Redox Signal 2008;10:287-01.

Brown AD. Microbial water stress physiology. Principles and perspectives. Inc: John Wiley & Sons; 1990.

Buenger J, Degwert J, Driller HJ. The protective function of compatible solute ectoine on the skin cells and its biomolecules with respect to UV radiation, immunosuppression and membrane damage. IFSCC Mag 2001;4:1–6.

Buenger J, Driller H. Ectoin: an effective natural substance to prevent UVA-induced premature photoaging. Skin Pharmacol Physiol 2004;17:232–7.

Bunger J. Ectoine added protection and care for the skin. Eur Cosmet 1999;7:22–4.

Buommino E, Schiraldi C, Baroni A, Paoletti I, Lamberti M, De Rosa M, et al. Ectoine from halophilic microorganisms induces the expression of hsp70 and hsp70B′ in human keratinocytes modulating the proinflammatory response. Cell Stress Chaperones 2005;10(3):197-03.

Burg MB, Ferraris JD. Intracellular organic osmolytes: function and regulation. J Biol Chem 2008;283:7309–13.

Bursy J, Kuhlmann AU, Pittelkow M, Hartmann H, Jebbar M, Pierik AJ, et al. Synthesis and uptake of the compatible solutes ectoine and 5-hydroxyectoine by *Streptomyces coelicolor* A3(2) in response to salt and heat stresses. Appl Environ Microbiol 2008;74:7286–96.

Bursy J, Pierik AJ, Pica N, Bremer E. Osmotically induced synthesis of the compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine hydroxylase. J Biol Chem 2007;282:31147–55.

Calderon MI, Vargas C, Rojo F, Iglesias-Guerra F, Csonka LN, Ventosa A, et al. Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043. Microbiology 2004;150:3051–63.

Canamas TP, Vinas I, Usall J, Magan N, Morello JR, Teixido N. Relative importance of amino acids, glycine-betaine and ectoine synthesis in the biocontrol agent *Pantoea agglomerans* CPA-2 in response to osmotic, acidic and heat stress. Lett Appl Microbiol 2007;45:6-12.

Canovas D, Borges N, Vargas C, Ventosa A, Nieto JJ, Santos H. Role of N gamma-acetyldiaminobutyrate as an enzyme stabilizer and an intermediate in the biosynthesis of hydroxyectoine. Appl Environ Microbiol 1999;65:3774–9.

Canovas D, Vargas C, Calderon MI, Ventosa A, Nieto JJ. Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. Syst Appl Microbiol 1998;21:487–97.

Canovas D, Vargas C, Iglesias-Guerra F, Csonka LN, Rhodes D, Ventosa A, et al. Isolation and characterization of salt-sensitive mutants of the moderate halophile *Halomonas elongata* and cloning of the ectoine synthesis genes. J Biol Chem 1997;272:25794-01.

Canovas M, Maiquez J, De Diego T, Buendia B, Espinosa G, Iborra JL. Membrane cell retention systems for continuous production of L-carnitine using *Proteus* sp. J Membr Sci 2003;214:101–11.

Cayley S, Lewis BA, Record Jr MT. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J Bacteriol 1992;174:1586–95.

Clegg JS, Seitz P, Seitz W, Hazlewood CF. Cellular responses to extreme water loss: the water-replacement hypothesis. Cryobiology 1982;19:306–16.

Cordingley MG, LaFemina RL, Callahan PL, Condra JH, Sardana VV, Graham DJ, et al. Sequence-specific interaction of Tat protein and Tat peptides with the transactivation-responsive sequence element of human immunodeficiency virus type 1 *in vitro*. Proc Natl Acad Sci USA 1990;87:8985–9.

Crowe JH, Carpenter JF, Crowe LM, Anchordoguy TJ. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 1990;43:219–31.

Cruz PE, Silva AC, Roldao A, Carmo M, Carrondo MJT, Alves PM. Screening of novel excipients for improving the stability of retroviral and adenoviral vectors. Biotechnol Prog 2006;22:568–76.

da Costa MS, Santos H, Galinski EA, Antranikian G. An overview of the role and diversity of compatible solutes in Bacteria and Archaea. Adv Biochem Eng Biotechnol 1998;61: 117–53.

Dennis PP, Shimmin LC. Evolutionary divergence and salinity-mediated selection in halophilic Archaea. Microbiol Mol Biol Rev 1997;61:90-04.

Dingwall C, Ernberg I, Gait MJ, Green SM, Heaphy S, Karn J, et al. Skinner MA.HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. EMBO J 1990;9:4145–53.

Dobson CM. Protein folding and disease: a view from the first Horizon Symposium. Nat Rev Drug Discov 2003;2:154–60.

Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, et al. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol 2005;2:10–9.

Fallet C, Rohe P, Franco-Lara E. Process optimization of the integrated synthesis and secretion of ectoine and hydroxyectoine under hyper/hypo osmotic stress. Biotechnol Bioeng 2010;107:124–33.

Fernandez-Linares L, Faure R, Bertrand JC, Gauthier M. Ectoine as the predominant osmolyte in the marine bacterium *Marinobacter hydrocarbonoclasticus* grown on eicosane at high salinities. Lett Appl Microbiol 1996;22:169–72.

Ferrigno P, Silver PA. Polyglutamine expansions: proteolysis, chaperones, and the dangers of promiscuity. Neuron 2000;26:9-12.

Fischel U, Oren A. Fate of compatible solutes during dilution stress in *Ectothiorhodospira marismortui*. FEMS Microbiol Lett 1993;113:113–8.

Fligiel SE, Varani J, Datta SC, Kang S, Fisher GJ, Voorhees JJ. Collagen degradation in aged/photodamaged skin *in vivo* and after exposure to matrix metalloproteinase-1 *in vitro*. J Invest Dermatol 2003;120:842–8.

Forloni G, Angeretti N, Chiesa R, Monzani E, Salmona M, Bugiani O, et al. Neurotoxicity of a prion protein fragment. Nature 1993;362:543–6.

Fourtanier A, Bernerd F, Bouillon C, Marrot L, Moyal D, Seite S. Protection of skin biological targets by different types of sunscreens. Photodermatol Photoimmunol Photomed 2006;22:22-2.

Frings E, Sauer T, Galinski EA. Production of hydroxyectoine-high cell-density cultivation and osmotic downshock of *Marinococcus* strain M52. J Biotechnol 1995;43:53-1.

Furusho K, Yoshizawa T, Shoji S. Ectoine alters subcellular localization of inclusions and reduces apoptotic cell death induced by the truncated Machado–Joseph disease gene product with an expanded polyglutamine stretch. Neurobiol Dis 2005;20:170–8.

Galinski EA, Herzog RM. The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). Arch Microbiol 1990;153:607–13.

Galinski EA, Pfeiffer HP, Trüper HG. 1, 4, 5, 6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid — a novel cyclic amino-acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira*. Eur J Biochem 1985;149:135–9.

Galinski EA, Stein M, Amendt B, Kinder M. The kosmotropic (structure-forming) effect of compensatory solutes. Comp Biochem Physiol 1997;117A:357–65.

Galinski EA, Trueper H, Sauer T, Trueper HG, Trüper HG, Galinski E; Bitop Ges Biotechnische Optimierung MBH assignee. 1994. Prodn. of natural substances, e.g. new hydroxy:ectoin deriv — by *in vitro* release from cells, esp. microorganisms, following application of stress patent DE4244580-A.

Garcia-Estepa R, Argandoña M, Reina-Bueno M, Capote N, Iglesias-Guerra F, Nieto JJ, et al. The ectD gene, which is involved in the synthesis of the compatible solute hydroxyectoine, is essential for thermoprotection of the halophilic bacterium *Chromohalobacter salexigens*. J Bacteriol 2006;188:3774–84.

Giannessi D, Caselli C, Vitale RL, Crucean A, Murzi B, Del Ry S, et al. A possible cardioprotective effect of heat shock proteins during cardiac surgery in pediatric patients. Pharmacol Res 2003;48:519–29.

Göller K, Galinski EA. Protection of a model enzyme (lactate dehydrogenase) against heat, urea and freeze–thaw treatment by compatible solutes additives. J Mol Catal B Enzym 1999;7:37–45.

Gonzalez-Ulloa M, Flores ES. Senility of the face. Basic study to understand its causes and effects. Plast Reconstr Surg 1965;36:239–46.

Graf R, Anzali S, Buenger J, Pfluecker F, Driller H. The multifunctional role of ectoine as a natural cell protectant. Clin Dermatol 2008;26:326–33.

Grammam K, Volke A, Kunte HJ. New type of osmoregulated solute transporter identified in halophilic members of the bacteria domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM 2581 (T). J Bacteriol 2002;184:3078–85.

Grammel N. Molekulargenetische und biochemische analyse der biosynthese von 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidin und seinem 5-hydroxyderivat, zwei salzstreβinduzierbaren osmolyten in *Streptomyces chrysomallus*. Berlin, Germany: Technische Universität Berlin; 2000.

Grant D, Wall W, Mimeault R, Zhong R, Ghent C, Garcia B, et al. Successful small-bowel/liver transplantation. Lancet 1990;335:181–4.

Grether-Beck S, Timmer A, Felsner I, Brenden H, Brammertz D, Krutmann J. Ultraviolet A-induced signaling involves a ceramide-mediated autocrine loop leading to ceramide de novo synthesis. J Invest Dermatol 2005;125:545–53.

Guzman H, Van-Thuoc D, Martin J, Hatti-Kaul R, Quillaguaman J. A process for the production of ectoine and poly(3-hydroxybutyrate) by *Halomonas boliviensis*. Appl Microbiol Biotechnol 2009;84:1069–77.

Hai T, Oppermann-Sanio FB, Steinbuchel A. Molecular characterization of a thermostable cyanophycin synthetase from the thermophilic cyanobacterium *Synechococcus* sp. strain MA19 and *in vitro* synthesis of cyanophycin and related polyamides. Appl Environ Microbiol 2002;68:93-101.

Harjes S, Scheidig A, Bayer P. Expression, purification and crystallization of human 3′-phosphoadenosine-5′-phosphosulfate synthetase 1. Acta Crystallogr D 2004;60:350–2.

Harper JD, Lansbury Jr PT. Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu Rev Biochem 1997;66:385-07.

He J, Huang X, Gu LF, Jiang JD, Li SP. Cloning of the ectoine biosynthesis gene ectABC from *Halomonas* sp. BYS-1 and salt stressed expression in *Escherichia coli*. Wei Sheng Wu Xue Bao 2006;46:28–32.

Heinrich U, Garbe B, Tronnier H. *In vivo* assessment of ectoin: a randomized, vehicle-controlled clinical trial. Skin Pharmacol Physiol 2007;20:211–8.

Inbar L, Lapidot A. The structure and biosynthesis of new tetrahydropyrimidine derivatives in actinomycin-D producer *Streptomyces parvulus*. Use of C-13-labeled and N-15-labeled glutamate and C-13 and N-15 NMR-spectroscopy. J Biol Chem 1988;263:16014–22.

Jebbar M, Sohn-Bosser L, Bremer E, Bernard T, Blanco C. Ectoine-induced proteins in *Sinorhizobium meliloti* include an ectoine ABC-type transporter involved in osmoprotection and ectoine catabolism. J Bacteriol 2005;187:1293-04.

Jebbar M, Talibart R, Gloux K, Bernard T, Blanco C. Osmoprotection of *Escherichia coli* by ectoine — uptake and accumulation characteristics. J Bacteriol 1992;174:5027–35.

Jeffries TW. Ethanol fermentation on the move. Nat Biotechnol 2005;23:40–1.

Joentgen W, Groth T, Steinbuchel A, Hai T, Oppermann FB; Bayer AG, assignee. Polyaspartic acid homopolymers and copolymers, biotechnological production and use thereof. 2001

Kakizuka A. Protein precipitation: a common etiology in neurodegenerative disorders? Trends Genet 1998;14:396-02.

Kanapathipillai M, Ku SH, Girigoswami K, Park CB. Small stress molecules inhibit aggregation and neurotoxicity of prion peptide 106–126. Biochem Biophys Res Commun 2008;365:808–13.

Kanapathipillai M, Lentzen G, Sierks M, Park CB. Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's beta-amyloid. FEBS Lett 2005;579: 4775–80.

Kanias T, Acker JP. Mammalian cell desiccation: facing the challenges. Cell Preserv Technol 2006;4:253–77.

Keshavarz K. Nonphytate phosphorus requirement of laying hens with and without phytase on a phase feeding program. Poult Sci 2000;79:748–63.

Knapp S, Ladenstein R, Galinski EA. Extrinsic protein stabilization by the naturally occurring osmolytes beta-hydroxyectoine and betaine. Extremophiles 1999;3:191–8.

Koichi M, Mitsuhiko M, Tatsuo N, Yoshio S; Takeda Chem Ind Ltd (Take), assignee. 1991. Preparation of tetrahydropyrimidine derivatives used for medical agent, etc., by reacting e.g. acetonitrile with e.g. methyl 2,4-diaminobutyrate. Japan patent JP3031265-A.

Kolp S, Pietsch M, Galinski EA, Gutschow M. Compatible solutes as protectants for zymogens against proteolysis. Biochim Biophys Acta 2006;1764:1234–42.

Kong SE, Blennerhassett LR, Heel KA, McCauley RD, Hall JC. Ischaemia–reperfusion injury to the intestine. Aust N Z J Surg 1998;68:554–61.

Krutmann J. Ultraviolet A radiation-induced biological effects in human skin: relevance for photoaging and photodermatosis. J Dermatol Sci 2000;23(Suppl 1):S22–6.

Krutmann J. The role of mitochondrial DNA mutations in photoaging of human skin: implications for the development of photoprotective strategies; 2003. London.

Kuhlmann AU, Bremer E. Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp. Appl Environ Microbiol 2002;68:772–83.

Kunte HJ. Osmoregulation in Bacteria: compatible solute accumulation and osmosensing. Environ Chem 2006;3:94–9.

Kurz M. Compatible solute influence on nucleic acids: many questions but few answers. Saline Sys 2008;4:6.

Lanyi JK. Salt dependent properties of proteins from extremely halophilic bacteria. Bacteriol Rev 1974;38:272–90.

Lapidot A, Ben-Asher E, Eisenstein M. Tetrahydropyrimidine derivatives inhibit binding of a Tat-like, arginine-containing peptide, to HIV TAR RNA *in vitro*. FEBS Lett 1995;367:33–8.

Lapidot A, Iakobashvili R, Malin G; Yeda Res & Dev Co Ltd (Yeda) Yeda Res&Dev Co Ltd (Yeda), assignee. 1999. Performing nucleic acid extension reaction in presence of osmoprotectant, e.g. proline, particularly for sequencing and amplification — increases stability of polymerase and reduces melting temperature of template or primer. Patent WO9941410-A; EP1053350-A; WO9941410-A1; EP1053350-A1; JP2002503482-W; US6428986-B1; IL137725-A; US7150980-B1; US2007212760-A1; US7387878-B2; EP1053350-B1; DE69940662-E; ES2323561-T3; JP4393700-B2.

Lee JC, Timasheff SN. The stabilization of proteins by sucrose. J Biol Chem 1981;256: 7193-01.

Lentzen G, Schwarz T. Extremolytes: natural compounds from extremophiles for versatile applications. Appl Microbiol Biotechnol 2006;72:623–34.
Le Rudulier D, Bouillard L. Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*. Appl Environ Microbiol 1983;46: 152–9.
Leung TKC, Rajendran MY, Monfries C, Hall C, Lim L. The human heat-shock protein family. Expression of a novel heat-inducible Hsp70 (Hsp70′) and isolation of its cDNA and genomic DNA. Biochem J 1990;267:125–32.
Lippert K, Galinski EA. Enzyme stabilization be ectoine-type compatible solutes: protection against heating, freezing and drying. Appl Microbiol Biotechnol 1992;37:61–5.
Liu Y, Bolen DW. The peptide backbone plays a dominant role in protein stabilization by naturally. Biochemistry 1995;3(4):12884–91.
Lizuka H, Ishida-Yamamoto A, Kajita S, Tsutsui M, Ohkuma N. Effects of UVB irradiation on epidermal adenylate cyclase responses *in vitro*. Arch Dermatol Res 1988;280: 163–7.
Lo CC, Bonner CA, Xie G, D'Souza M, Jensen RA. Cohesion group approach for evolutionary analysis of aspartokinase, an enzyme that feeds a branched network of many biochemical pathways. Microbiol Mol Biol Rev 2009;73:594-51.
Louis P, Galinski EA. Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiology 1997;143:1141–9.
Louis P, Trüper HG, Galinski EA. Survival of *Escherichia coli* during drying and storage in the presence of compatible solutes. Appl Microbiol Biotechnol 1994;41:684–8.
Macnee W. Pathogenesis of chronic obstructive pulmonary disease. Clin Chest Med 2007;28:479-13.
Malin G, Iakobashvili R, Lapidot A. Effect of tetrahydropyrimidine derivatives on protein–nucleic acids interaction. J Biol Chem 1999;274:6920–9.
Malin G, Lapidot A. Induction of synthesis of tetrahydropyrimidine derivatives in *Streptomyces*. J Bacteriol 1996;178:385–95.
Manzanera M, de Castro AG, Tondervik A, Rayner-Brandes M, Strom AR, Tunnacliffe A. Hydroxyectoine is superior to trehalose for anhydrobiotic engineering of *Pseudomonas putida* KT2440. Appl Environ Microbiol 2002;68:4328–33.
Manzanera M, Vilchez S, Tunnacliffe A. High survival and stability rates of *Escherichia coli* dried in hydroxyectoine. FEMS Microbiol Lett 2004a;233:347–52.
Manzanera M, Vilchez S, Tunnacliffe A. Plastic encapsulation of stabilized *Escherichia coli* and *Pseudomonas putida*. Appl Environ Microbiol 2004b;70:3143–5.
Mascellani N, Liu X, Rossi S, Marchesini J, Valentini D, Arcelli D, et al. Compatible solutes from hyperthermophiles improve the quality of DNA microarrays. BMC Biotechnol 2007;7:82.
Maskow T, Babel W. Calorimetrically obtained information about the efficiency of ectoine synthesis from glucose in *Halomonas elongata*. Biochim Biophys Acta 2001;1528: 60–70.
Maskow T, Kleinsteuber S. Carbon and energy fluxes during haloadaptation of *Halomonas* sp. EF11 growing on phenol. Extremophiles 2004;8:133–41.
Moghaieb REA, Tanaka N, Saneoka H, Murooka Y, Ono H, Morikawa H, et al. Characterization of salt tolerance in ectoine-transformed tobacco plants (*Nicotiana tabaccum*): photosynthesis, osmotic adjustment, and nitrogen partitioning. Plant Cell Environ 2006;29:173–82.
Morbach S, Krämer R. Body shaping under water stress: osmosensing and osmoregulation of solute transport in bacteria. Chembiochem 2002;3:384–97.
Motitschke (LH, DE), Driller, Hansjurgen (Monheim, DE), Galinski, Erwin (Bonn, DE); Merck Patent GmbH (DE), assignee. 2000. Ectoin and ectoin derivatives as moisturizers in cosmetics. United States.
Murphy RM. Peptide aggregation in neurodegenerative disease. Annu Rev Biomed Eng 2002;4:155–74.
Nagata S, Maekawa Y, Ikeuchi T, Wang YB, Ishida A. Effect of compatible solutes on the respiratory activity and growth of *Escherichia coli* K-12 under NaCl stress. J Biosci Bioeng 2002;94:384–9.
Nagata S, Wang Y, Oshima A, Zhang L, Miyake H, Sasaki H, et al. Efficient cyclic system to yield ectoine using *Brevibacterium* sp. JCM 6894 subjected to osmotic downshock. Biotechnol Bioeng 2008;99:941–8.
Nagata S, Wang YB. Accumulation of ectoine in the halotolerant *Brevibacterium* sp. JCM 6894. J Biosci Bioeng 2001;91:288–93.
Nakayama H, Yoshida K, Ono H, Murooka Y, Shinmyo A. Ectoine, the compatible solute of *Halomonas elongata*, confers hyperosmotic tolerance in cultured tobacco cells. Plant Physiol 2000;122:1239–47.
Naughton LM, Blumerman SL, Carlberg M, Boyd EF. Osmoadaptation among *Vibrio* species and unique genomic features and physiological. Appl Environ Microbiol 2009;75:2802–10.
Obon JM, Maiquez JR, Canovas M, Kleber HP, Iborra JL. L(−)-Carnitine production with immobilized *Escherichia coli* cells in continuous reactors. Enzyme Microb Technol 1997;21:531–6.
Obon JM, Maiquez JR, Canovas M, Kleber HP, Iborra JL. High-density *Escherichia coli* cultures for continuous L(−)-carnitine production. Appl Microbiol Biotechnol 1999;51:760–4.
Omata Y, Lewis JB, Rotenberg S, Lockwood PE, Messer RLW, Noda M, et al. Intra- and extracellular reactive oxygen species generated by blue light. J Biomed Mater Res 2006;77:470–7.
Ono H, Okuda M, Tongpim S, Imai K, Shinmyo A, Sakuda S, et al. Accumulation of compatible solutes, ectoine and hydroxyectoine, in a moderate halophile, *Halomonas elongata* KS3 isolated from dry salty land in Thailand. J Ferment Bioeng 1998;85:362–8.
Onraedt AE, Walcarius BA, Soetaert WK, Vandamme EJ. Optimization of ectoine synthesis through fed-batch fermentation of *Brevibacterium epidermis*. Biotechnol Progr 2005;21:1206–12.
Oren A. Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 1999;63:334–48.
Oren A, Heldal M, Norland S, Galinski EA. Intracellular ion and organic solute concentrations of the extremely halophilic bacterium *Salinibacter ruber*. Extremophiles 2002;6:491–8.
Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, et al. Conversion of alpha-helices into beta-sheets features in the formation of the. Proc Natl Acad Sci USA 1993;90:10962–6.
Paulson HL. Protein fate in neurodegenerative proteinopathies: polyglutamine diseases join. Am J Hum Genet 1999;64:339–45.
Perutz MF. Glutamine repeats and neurodegenerative diseases: molecular aspects. Trends Biochem Sci 1999;24:58-3.
Peters A, von Klot S, Heier M, Trentinaglia I, Hormann A, Wichmann HE, et al. Exposure to traffic and the onset of myocardial infarction. N Engl J Med 2004;351:1721–30.
Peters P, Galinski EA, Trüper HG. The biosynthesis of ectoine. FEMS Microbiol Lett 1990;71:157–62.
Pflaum M, Kielbassa C, Garmyn M, Epe B. Oxidative DNA damage induced by visible light in mammalian cells: extent. Mutat Res 1998;408:137–46.
Pfluecker F, Bunger J, Hitzel S, Witte G, Beck J, Lergenmuller M, et al. Complete photo protection — going beyond visible endpoints. SOWF J 2005;131:20–30.
Pflughoeft KJ, Kierek K, Watnick PI. Role of ectoine in *Vibrio cholerae* osmoadaptation. Appl Environ Microbiol 2003;69:5919–27.
Poolman B, Glaasker E. Regulation of compatible solute accumulation in bacteria. Mol Microbiol 1998;29:397-07.
Prabhu J, Schauwecker F, Grammel N, Keller U, Bernhardt M. Functional expression of the ectoine hydroxylase gene (thpD) from *Streptomyces chrysomallus* in *Halomonas elongata*. Appl Environ Microbiol 2004;70:3130–2.
Pul U, Wurm R, Wagner R. The role of LRP and H-NS in transcription regulation: involvement of synergism. J Mol Biol 2007;366:900–15.
Qu Y, Bolen CL, Bolen DW. Osmolyte-driven contraction of a random coil protein. Proc Natl Acad Sci USA 1998;95:9268–73.
Rabe JH, Mamelak AJ, McElgunn PJS, Morison WL, Sauder DN. Photoaging: mechanisms and repair. J Am Acad Dermatol 2006;55:1-19.
Rai M, Pal M, Sumesh KV, Jain V, Sankaranarayanan A. Engineering for biosynthesis of ectoine (2-methyl 4-carboxy tetrahydro pyrimidine) in tobacco chloroplasts leads to accumulation of ectoine and enhanced salinity tolerance. Plant Sci 2006;170: 291-06.
Rajan LA, Joseph TC, Thampuran N, James R, Kumar KA, Viswanathan C, et al. Cloning and heterologous expression of ectoine biosynthesis genes from *Bacillus halodurans* in *Escherichia coli*. Biotechnol Lett 2008;30:1403–7.
Raymond JC, Sistrom WR. *Ectothiorhodospira halophila* — a new species of genus *Ectothiorhodospira*. Arch Mikrobiol 1969;69:121–6.
Reed RH, Warr SRC, Kerby NW, Stewart WDP. Osmotic shock induced release of low molecular weight metabolites from free living and immobilized cyanobacteria. Enzyme Microb Technol 1986;8:101–4.
Riesenberg D, Guthke R. High-cell-density cultivation of microorganisms. Appl Microbiol Biotechnol 1999;51:422–30.
Rimsky S, Zuber F, Buckle M, Buc H. A molecular mechanism for the repression of transcription by the H-NS protein. Mol Microbiol 2001;42:1311–23.
Roberts MF. Osmoadaptation and osmoregulation in archaea. Front Biosci 2000;5:D796–812.
Roberts MF. Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Systems 2005;1:5.
Roessler M, Müller V. Osmoadaptation in Bacteria and Archaea: common principles and differences. Environ Microbiol 2001;3:743–54.
Roy S, Delling U, Chen CH, Rosen CA, Sonenberg N. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated. Genes Dev 1990;4:1365–73.
Sander CS, Chang H, Hamm F, Elsner P, Thiele JJ. Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. Int J Dermatol 2004;43:326–35.
Sauer T, Galinski EA. Bacterial milking: a novel bioprocess for production of compatible solutes. Biotechnol Bioeng 1998;57:306–13.
Saum SH, Müller V. Growth phase-dependent switch in osmolyte strategy in a moderate halophile: ectoine is a minor osmolyte but major stationary phase solute in *Halobacillus halophilus*. Environ Microbiol 2008;10:716–26.
Schiraldi C, De Rosa M. The production of biocatalysts and biomolecules from extremophiles. Trends Biotechnol 2002;20:515–21.
Schiraldi C, Maresca C, Catapano A, Galinski EA, De Rosa M. High-yield cultivation of *Marinococcus* M52 for production and recovery of hydroxyectoine. Res Microbiol 2006;157:693–9.
Schmitz RPH, Galinski EA. Compatible solutes in luminescent bacteria of the genera *Vibrio*, *Photobacterium* and *Photorhabdus* (*Xenorhabdus*): occurrence of ectoine, betaine and glutamate. FEMS Microbiol Lett 1996;142:195-01.
Schnoor M, Voss P, Cullen P, Boking T, Galla HJ, Galinski EA, et al. Characterization of the synthetic compatible solute homoectoine as a potent PCR. Biochem Biophys Res Commun 2004;322:867–72.
Schroder O, Wagner R. The bacterial regulatory protein H-NS — a versatile modulator of nucleic acid. Biol Chem 2002;383:945–60.
Schubert T, Maskow T, Benndorf D, Harms H, Breuer U. Continuous synthesis and excretion of the compatible solute ectoine by a transgenic, nonhalophilic bacterium. Appl Environ Microbiol 2007;73:3343–7.
Schwamborn M. Chemical synthesis of polyaspartates: a biodegradable alternative to currently used polycarboxylate homo- and copolymers. Polym Degrad Stab 1998;59: 39-5.
Severin J, Wohlfarth A, Galinski EA. The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J Gen Microbiol 1992;138:1629-8.
Shiloach J, Fass R. Growing *E. coli* to high cell density — a historical perspective on method development. Biotechnol Adv 2005;23:345–57.

Sideris EG, Papageorgiou GC, Charalampous SC, Vitsa EM. A spectrum response study on single strand DNA breaks, sister chromatid. Pediatr Res 1981;15:1019–23.
Souil E, Capon A, Mordon S, Dinh-Xuan AT, Polla BS, Bachelet M. Treatment with 815-nm diode laser induces long-lasting expression of 72-kDa heat. Br J Dermatol 2001;144:260–6.
Stahl CH, Roneker KR, Thornton JR, Lei XG. A new phytase expressed in yeast effectively improves the bioavailability of phytate phosphorous to weanling pigs. J Anim Sci 2000;78:668–74.
Sydlik U, Gallitz I, Albrecht C, Abel J, Krutmann J, Unfried K. The compatible solute ectoine protects against nanoparticle-induced neutrophilic. Am J Respir Crit Care Med 2009;180:29–35.
Talibart R, Jebbar M, Gouesbet G, Himdikabbab S, Wroblewski H, Blanco C, et al. Osmoadaptation in rhizobia — ectoine-induced salt tolerance. J Bacteriol 1994;176: 5210–7.
Tavaria M, Gabriele T, Kola I, Anderson RL. A hitchhiker's guide to the human Hsp70 family. Cell Stress Chaperones 1996;1:23–8.
Tetsch L, Kunte HJ. The substrate-binding protein TeaA of the osmoregulated ectoine transporter TeaABC from *Halomonas elongata*: purification and characterization of recombinant TeaA. FEMS Microbiol Lett 2002;211:213–8.
Timasheff SN. Protein hydration, thermodynamic binding, and preferential hydration. Biochemistry 2002;41:13473–82.
Tolmasoff JM, Ono T, Cutler RG. Superoxide dismutase: correlation with life-span and specific metabolic rate in primate species. Proc Natl Acad Sci USA 1980;77:2777–81.
Tsapis A, Kepes A. Transient breakdown of permeability barrier of membrane of *Escherichia coli* upon hypoosmotic shock. Biochim Biophys Acta 1977;469:1-12.
Ullah AH. Production, rapid purification and catalytic characterization of extracellular. Prep Biochem 1988;18:443–58.
Van-Thuoc D, Guzman H, Guillaguaman J, Hatti-Kaul R. High productivity of ectoines by *Halomonas boliviensis* using a combined two-step fed-batch culture and milking process. J Biotechnol 2010;147:46–51.
Van-Thuoc D, Guzman H, Thi-Hang M, Hatti-Kaul R. Ectoine production by *Halomonas boliviensis*: optimization using response surface methodology. Mar Biotechnol (NY) 2009. doi:10.1007/s10126-009-9246-6.
Vargas C, Jebbar M, Carrasco R, Blanco C, Calderon MI, Iglesias-Guerra F, et al. Ectoines as compatible solutes and carbon and energy sources for the halophilic bacterium *Chromohalobacter salexigens*. J Appl Microbiol 2006;100:98-07.
Ventosa A, Nieto JJ, Oren A. Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 1998;62:504–44.
Vermeulen V, Kunte HJ. *Marinococcus halophilus* DSM 20408T encodes two transporters for compatible solutes belonging to the betaine-carnitine-choline transporter family: identification and characterization of ectoine transporter EctM and glycine betaine transporter BetM. Extremophiles 2004;8:175–84.
Wang A, Bolen DW. A naturally occurring protective system in urea-rich cells: mechanism of osmolyte. Biochemistry 1997;36:9101–8.
Wang SQ, Setlow R, Berwick M, Polsky D, Marghoob AA, Kopf AW, et al. Ultraviolet A and melanoma: a review. J Am Acad Dermatol 2001;44:837–46.
Wang Y, Zhang L. Ectoine improves yield of biodiesel catalyzed by immobilized lipase. J Mol Catal Enzym 2010;62:90–5.
Wei L, Wedeking A, Buttner R, Kalff JC, Tolba RH, van Echten-Deckert G. A natural tetrahydropyrimidine protects small bowel from cold ischemia and. Pathobiology 2009;76:212–20.
Welsh DT. Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol Rev 2000;24:263–90.
Wiggins PM. Role of water in some biological processes. Microbiol Rev 1990;54:432–49.
Wohlfarth A, Severin J, Galinski EA. The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family *Halomonadaceae*. J Gen Microbiol 1990;136:705–12.
Yancey PH. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 2005;208:2819–30.
Yu I, Jindo Y, Nagaoka M. Microscopic understanding of preferential exclusion of compatible solute ectoine. J Phys Chem B 2007;111:10231–8.
Yu I, Nagaoka M. Slowdown of water diffusion around protein in aqueous solution with ectoine. Chem Phys Lett 2004;388:316–21.
Zhang L, Lang Y, Wang C, Nagata S. Promoting effect of compatible solute ectoine on the ethanol fermentation by *Zymomonas mobilis* CICC10232. Proc Biochem 2008;43:642–6.
Zhang L, Wang Y, Zhang C, Wang Y, Zhu D, Wang C, et al. Supplementation effect of ectoine on thermostability of phytase. J Biosci Bioeng 2006;102:560–3.
Zhang LH, Lang YJ, Nagata S. Efficient production of ectoine using ectoine-excreting strain. Extremophiles 2009;13:717–24.
Zou Y, Hernandez F, Burgos E, Martinez L, Gonzalez-Reyes S, Fernandez-Dumont V, et al. Bacterial translocation in acute rejection after small bowel transplantation in rats. Pediatr Surg Int 2005;21:208–11.