Industrial Production of the Cell Protectant Ectoine: Protection Mechanisms, Processes, and Products

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Abstract: *Bacteria*, *Archaea* and *Eukarya* can adapt to saline environments by accumulating compatible solutes in order to maintain an osmotic equilibrium. Compatible solutes are of diverse chemical structure (sugars, polyols, amino acid derivatives) and are beneficial for bacterial cells not only as osmoregulatory solutes, but also as protectants of proteins by mitigating detrimental effects of freezing, drying and high temperatures. The aspartate derivative ectoine is a wide spread compatible solute in *Bacteria* and possesses additional protective properties compared with other compatible solutes, and stabilizes even whole cells against stresses such as UV radiation or cytotoxins. The protective properties of ectoine for proteins can be explained by its strong (kosmotropic) interaction with water and subsequent exclusion from protein surface, the decrease of the solubility of the peptide backbone and the strengthening of intramolecular hydrogen bonds (secondary structures). The stabilizing and UV-protective properties of ectoine attracted industry, which saw the potential to market ectoine as a novel active component in health care products and cosmetics. In joint efforts of industry and research large-scale fermentation procedures have been developed with the halophilic bacterium *Halomonas elongata* used as a producer strain. The two key technologies that allow for the annual production of ectoine on a scale of tons are the bacterial milking procedure and the development and application of ectoine-excreting mutants ("leaky" mutant). The details of these two procedures including the strain development and fermentation processes will be introduced and current and future applications of ectoine will be discussed.

Keywords: Bacterial milking, batch fermentation, continuous culture, ectoine excretion, Hofmeister effect, kosmotrope, "leaky" mutant, osmophobic effect, protein protection, preferential exclusion.

INTRODUCTION

Hypersaline environments with salt concentrations up to saturation of sodium chloride (approximately 300 g L^{-1}) are mainly inhabited by microorganisms. Many hypersaline environments derived from seawater by evaporation and their ion composition are therefore similar to seawater. These so-called thalassohaline environments comprise sodium and chloride as their main ions and have a neutral to slightly alkaline pH. Typical thalassohaline habitats are solar saltern crystallizer ponds, which can be found in tropical and subtropical areas around the world or the Great Salt Lake in Utah [1]. Athalassohaline hypersaline habitats exhibit a quite different pH and ion composition and in the case of the Dead Sea divalent cations such as Mg^{2+} and Ca^{2+} are dominating. Athalassohaline and thalassohaline environments provide harsh living conditions; however, both habitats harbour halophilic microorganisms, or halophiles, from all three domains of life: *Archaea*, *Bacteria* and *Eukarya*. Halophilic microorganisms have developed two basically different mechanisms to cope with ionic strength and the considerable water stress, namely the "salt-in-cytoplasm" mechanism and the organic-osmolyte mechanism. Organisms following the salt-in-cytoplasm mechanism adapt the interior protein chemistry of the cell to high salt concentration [2]. The osmotic adjustment of the cell can be achieved by raising the salt concentration (KCl) in the cytoplasm according to the environmental osmolarity [3]. In contrast, microorganisms applying the organic-osmolyte strategy keep their cytoplasm, to a large extent, free of KCl and the design of the cell's interior remains basically unchanged. Instead, organisms of this group accumulate uncharged, highly water-soluble, organic compounds in order to maintain an osmotic equilibrium with the surrounding medium. The organic-osmolyte mechanism is widespread among *Bacteria* and *Eukarya* and also present in some methanogenic *Archaea* [4, 5]. Organic osmolytes are of diverse chemical structure comprising different types of sugars (e.g. trehalose), polyols, amino acids (proline) and their derivatives (ectoine, glycine-betaine) and are accumulated inside the cell either by *de novo* synthesis or by uptake from the surrounding environment. These non-ionic, highly water-soluble molecules do not disturb the metabolism, even at high cytoplasmic concentrations, and are thus aptly named compatible solutes [6]. Compatible solutes are beneficial for bacterial cells not only as osmoregulatory solutes, but also as

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protectants of proteins by mitigating detrimental effects of freezing, drying and high temperatures [7]. Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) [8], which is a widespread compatible solute in *Bacteria* [9-11], possesses additional protective properties compared with other zwitterionic compatible solutes such as glycine betaine and proline, and stabilizes even whole cells against stresses such as UV radiation, inflammation caused by nanoparticles or cytotoxins [12-17]. The properties of ectoine attracted industry, which saw the potential to put a novel protective compound for health care products on the market. Ectoine can be synthesized chemically [18, 19] but not for an acceptable price due to the high costs for the precursors. This led to the development of a more competitive biotechnological production of ectoine with halophilic bacteria. Halophilic microorganisms have been used for food fermentation in the Far East since centuries and are of importance for food industry in the production of flavouring agents [20]. However, besides food industry there are only a few biotechnical applications with halophiles that are successfully in use with industry. Most biotechnological applications exploiting halophiles and their products are still under development such as the application of bacteriorhodopsin as a holographic storage material, the production of poly-β-hydroxyalkanoate and extracellular polysaccharides by *Haloferax mediterranei*, and novel enzymes (e.g. restriction enzymes, proteases) from different halophilic *Archaea* and *Bacteria*. The only commercially successful applications with halophiles today are the production of β-carotene and ectoines. The β-carotene is produced by different species of the unicellular green alga *Dunaliella* and the first biotechnical production plant was already operable in the late 1960s in the former USSR [21]. Today, β-carotene is produced around the world [22] and is used as a food-coloring agent, as pro-vitamin A (retinol) and as an additive in cosmetics. The second biological production process with halophiles that is successful in the market is the above-mentioned production of the cell protectant ectoine. Ectoine is synthesized on a scale of tons by the company bitop (Witten, Germany) in a process with the halophilic γ-proteobacterium *H. elongata* used as producer strain (Fig. **1**). Its protective properties as stabilizer of enzymes and whole cells make ectoine a valuable compound, which is marketed in health care products and cosmetics.

Our purpose here is to review different aspects of the biotechnology of ectoine. In particular the present paper will

Fig. (1). Bioreactor at the bitop company for the large-scale production of ectoine with the halophilic producer strain *H. elongata.*

illustrate the mechanism of ectoine in stabilizing biological structures, explain the development of producer strains and their use in fermentation processes, and describe current and future applications of ectoine.

THE STABILIZATION PHENOMENON OF ECTOINES

Ectoines like other compatible solutes are selected by nature as a protection against environmental stresses. As such they also improve the stability of biomolecules. Protein and enzyme-stabilizing phenomena of ectoine have been reported by many authors and been included in reviews on cell stress protection [23, 24]. Here, it is our intention to go beyond a simple description of effects, but to depict the molecular interaction of ectoine with biomolecules, in particular proteins, and explain the underlying principles, which make it an excellent stabilizing solute. In order to achieve this, ectoine must be seen in context with other natural stabilizing compounds, the most prominent of which are N-methylated derivatives such as trimethylamine N-oxide (TMAO), glycine betaine, sarcosine, proline, sugars and polyols. Although one needs to keep in mind that stabilization phenomena reported by different authors might, to a certain degree, be specific for the system under investigation, we will try and extract general properties which are (more or less) characteristic for all compatible solutes and may serve to understand and predict the stabilizing effect of ectoines.

The Hofmeister Effect

The process of explaining the molecular interaction of compatible solutes has been influenced by attempts to explain the Hofmeister effect - and vice versa. The well-known salting-out effects in the Hofmeister series of inorganic salts on proteins (mainly controlled by the anion) has been known for more than 100 years now. At first, increase in surface tension has been suspected as a major factor for protein stabilization. This would explain the salting-out effect of Hofmeister salts, at least semi-quantitatively, by the work required of forming a cavity in a liquid as the product of surface area and surface tension [25, 26]. This first approach however proved too simplistic, as all inorganic salts tested (even denaturing NaSCN) increase surface tension. It was then concluded that the stabilizing effect caused by a positive surface tension increment must be partially counteracted by a second denaturing effect. The prevailing view with Hofmeister salts at present is that the binding of anions to the amide moieties (peptide bonds) is the counteracting force and that this effects is influenced by the polarizability of the anion, i.e. ability to shed their hydration shell [27]. This ability, typical for chaotropic anions, correlates with their octanol-water partition coefficient and characterizes the ability to decrease the surface tension at an oil-water interfaces (even though it may be increased at the air-water interface).

With compatible solutes, the good correlation between stabilization and increase in surface tension, in particular with compounds carrying hydroxyl groups (polyols and sugars), was at first taken as an indication for the importance of this force. However, looking at a wider range of osmolytes it also became apparent that surface tension does not correlate with stabilizing properties of compatible solutes either, as proline and TMAO for example have negative, and urea a positive surface tension increment [28]. From this it has become clear that surface tension, a property relating to the air-water interface, is not a good predictor of the interfacial tension at the protein-solution interface, which has a much higher dielectric constant than air. Therefore, in line with the conclusions from work on Hofmeister salts, it was predicted that specific hydration of solutes and possible interactions in terms of binding to and repulsion by surface-exposed groups of the protein also have to be considered [29].

Preferential Exclusion

The pioneering work of the Timasheff group of the 1980s has shown experimentally (dialysis experiments, density measurements) that the compatible solutes under investigation, often referred to as protecting osmolytes, were preferentially excluded from a protein´s hydration shell. They stabilize the native folded state relative to the unfolded denatured state because the free energy difference for denaturation (ΔG_{denat}) is raised as a consequence of a more unfavourable interaction of the solute-containing solvent with the unfolded state. Due to their preferential exclusion near to the protein surface, the phenomenon of protein stabilization could therefore be explained as the result of the tendency to minimize the surface area [30-32]. Although ectoines were not included at the time of the study, later work, using different techniques, has confirmed the exclusion phenomenon for ectoine [33]. However, which forces are responsible for the observed exclusion and subsequent stabilization is only slowly becoming clear.

Water Relations

Stabilizing/destabilizing effects of compatible solutes have also been related to a water-structuring effect induced by their presence. Solutes have been divided into water-structure makers (kosmotropes) and water-structure breakers (chaotropes). Although a strong effect of solutes on hydrogen bonding in the immediate hydration shell can be reasonably expected, a long-range influence on the hydrogen bonding network of the bulk has not been proven. Therefore, a kosmotrope may be seen as a solute which, by virtue of binding water more firmly in its own hydration shell than in bulk, increases the overall strength of hydrogen bonding in the system. Chaotropes, on the other hand, will disturb the hydrogen bonding network of the bulk (as do kosmotropes) without the formation of a strongly bound hydration shell in its immediate vicinity. As a consequence, bulk water will repell chaotropic solutes towards interfaces (such as protein-water interface) and keep kosmotropic solutes in the bulk. Using near-infrared (NIR) difference spectroscopy, a comparison of the self-hydration effects of betaine with that of other compatible solutes, in particular ectoine, has confirmed strong hydrogen shells of similar magnitude [34]. Therefore, a structuring effect of water in its vicinity (more than in bulk) appears to be a characteristic of compatible solutes including ectoines. This scenario is very similar to what has been suggested for stabilizing ions of the Hofmeister series [25] and helps to understand the

preferential exclusion phenomenon of osmolytes, which is in line with the view of Wiggins [35, 36] who also suggested a preference of compatible solutes for bulk water.

Osmophobic Effect

The work of the Bolen group has identified the peptide backbone as the major player for binding/repulsion effects, and subsequent protein stabilization [37, 38] and named this phenomenon the osmophobic effect [39]. Using diketo-piperazine, a cyclic dimer of glycine, as model compound for the peptide backbone, they were able to demonstrate that all compatible solutes tested were characterized by a positive transfer free energy (ΔG_{tr}) of backbone into osmolyte solution. Subsequently an apparent correlation was observed between measured ΔG_{tr} and fractional polar surface of the compatible solute. This allowed to establish a model in which the interaction energy of the solute with the peptide backbone depended on polarity and number of energetically equivalent ways to realize that interaction (degeneracy), i.e. the fractional polar surface area [40]. Despite the simplicity of this approach, neglecting other types of interaction affecting protein stability, the model correlated very well with experimental values of ΔG_{tr}, and emphasized the validity of the underlying concept that the osmolyte effect on proteins depends primarily on the osmolyte´s relative polarity and surface area (Fig. **2**). As osmolyte backbone interactions become increasingly favourable with osmolytes becoming more polar, the stabilizing properties of an osmolyte are predicted to improve with increasing hydrophobic surface area while at the same time still maintaining solubility in water. The quaternary ammonium group (as in TMA, betaine etc.), but also the 2-methyl pyrimidine group of ectoines, seem to be especially important as they convey a hydrophobic increment while at the same time avoiding hydrophobic fusion due to the repulsion caused by the positive charge. Therefore, the transfer free energy approach may hold as a suitable descriptor for interfacial free energies at the protein-solution interface [40].

Unfortunately ectoines were never included in the studies of the Bolen group. Unpublished results of the Galinski group, using diketopiperazine (DKP) as a model compound as proposed by Liu and Bolen (1995), have confirmed this universal stabilizing trend also for ectoine. Although the method of quantification (isocratic HPLC) was probably less accurate than the technique applied by Liu and Bolen [37], who applied precise density measurements using an Anton-Parr densimeter, the ΔG_{tr} energy obtained for ectoine was very similar to that of glycine betaine and fitted well onto the trend line. Therefore, it is made very likely that protein stabilization by ectoines is based on similar molecular mechanisms as those described for betaines (Fig. **2**).

It has subsequently been revealed that contribution of side chains to protein stability in osmolyte solution is not completely irrelevant, albeit much less important than the backbone [38, 41]. Additivity of the transfer model has also

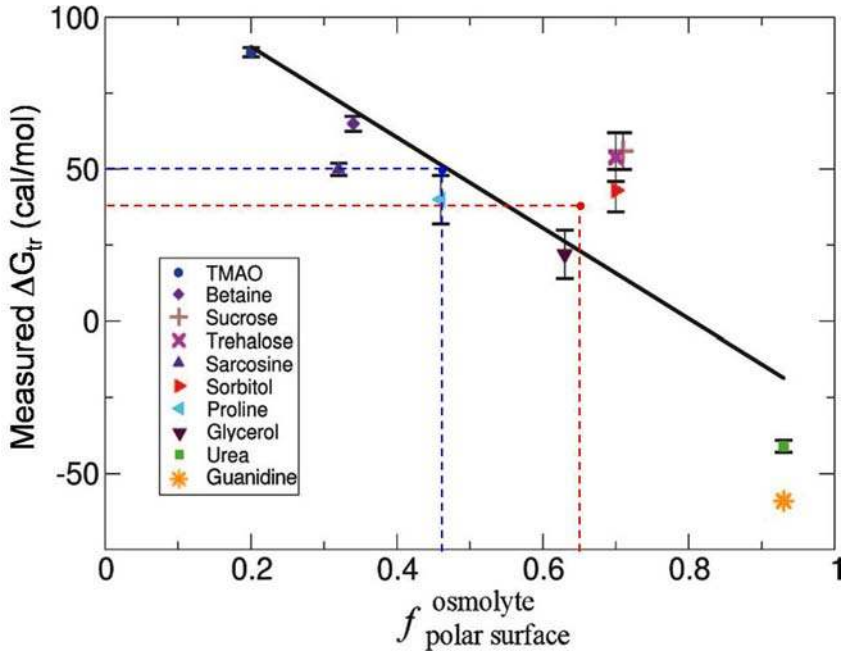

Fig. (2). Correlation of fractional polar surface of osmolyte with measured ΔG_{tr} values. Fractional polar surface area (*f* osmolyte polar surface) is plotted against measured ΔG_{tr} values for various osmolytes. The linear regression line has a negative slope with a correlation coefficient of 0.81, indicating that backbone / osmolyte interactions become increasingly favourable as osmolytes become increasingly polar (from (40): Street *et al*. (2006) PNAS 103: 13997-14002 ; corrections 17064 with permission, Copyright (2006) by National Academy of Sciences, USA). The figure was emended with fractional polar surface data for ectoine (0.46) and hydroxyectoine (0.64) determined as suggested by Street *et al*. (2006) and correlated with their backbone transfer free energy ΔGtr (50 cal/mol and 40 cal/mol, respectively) obtained from solubility studies with model compound diketopiperazine. From this it is apparent that ectoine (broken blue line) conforms to the trend line for typical osmolytes, whereas the considerably more polar hydroxyectoine (dotted red line) deviates from this norm and clusters with polyol and sugar-type compounds.

been tested using molecular dymanics simulations, which confirmed a linear increase in transfer free energy changes with increasing peptide chain length [42]. On the basis of these observations one can conclude that compatible solutes modulate the properties of the solvent water in such a way that it becomes a poorer solvent for the peptide backbone. By virtue of reducing its interaction with the solvent, intra-chain hydrogen bondings are strengthened and secondary structures stabilized [43]. As the α-helical core in particular resembles a conformation in which exposure of the backbone to the solvent is minimal, one would expect compatible solutes to strengthen this formation. This concept has gained support by the finding of Bourot *et al*. [44] that a metabolically inactive diaminopimelate decarboxylase (Ser 384 Phe mutation) is reactivated in the presence of compatible solutes like glycine betaine *in vitro* and *in vivo*. As demonstrated by the authors, the molecular cause for inactivity can be traced to a loss of α-helical structure, which is apparently recovered in the presence of compatible solutes. The authors conclude that compatible solutes may assist protein folding in a "chaperone-like" manner. Similarly, in a study on alanine-based model peptides, trimethylamine-N-oxide (TMAO) excelled as an osmolyte which induced helix formation [45] as predicted by the osmophobic effect hypothesis. In addition, it was observed that β-strands of α-chymotrypsin were enhanced in the presence of N-methylated ammonium type osmolytes [46].

It is our opinion that compatible solutes may be best described as low-molecular mass solutes enhancing the stability of secondary structures of the peptide backbone ("backbone chaperones"). Although ectoines have so far not been included in such studies, we expect that ectoine will exert similar effects to those observed for compatible solutes with N-methylated ammonium groups (e.g. TMAO, betaine, sarcosine etc.). To what extent this also applies to α-helix and β-sheet formation and whether one of the two conformations would be favoured in an equlibrium situation still awaits further investigations. Preliminary results on protein aggregation studies seem to indicate that compatible solutes (including ectoine) may also prevent pathogenic protein aggregation [15] and in particular β-sheet based amyloid formation as observed in neurodegenerative desease [47].

Atomic Force Microscopy (AFM) Studies

The general tendency of compatible solutes to enhance intramolecular forces and "stiffen" the structure of a protein has been confirmed by atomic force microscopy. Using fibronectin as a model revealed a marked influence of ectoine on the molecules persistence length, which is a measure of flexibility [48]. Ectoine´s influence on mechanical properties of polypeptides, i.e. the tendency to strengthen the compact coiled conformation, was also confirmed for the membrane protein bacteriorhodopsin [49].

Molecular Dynamics Simulations

Molecular dynamics simulations have mostly been perfomed with TMAO *vs* urea, and to a lesser extent with glycine betaine. Both trimethylated compatible solutes seem to behave in a similar way with water. This is reflected in their molecular structure comprising a hydrophilic region (the oxo groups) and a region of rather attenuated hydrophobicity. The simulations show net enhancement of water structure by TMAO (stronger water hydrogen bonds and spatial ordering of the solvent). It was concluded that this discourages interaction with the peptide backbone, as manifested in positive transfer free engergy [50]. A modelling study with TMAO (*vs* urea) and chymotrypsin inhibitor 2 (CI2), a small globular protein, seems to indicate that enhancement of the solvent structure in the hydration shell of TMAO prevents the initial attack of water molecules (i.e. competition with intramolecular H bonds) to dissolve the peptide core [51]. Similar studies modeling the effect of ectoine on CI2 [33] have come to the following conclusions: ectoine molecules are also preferentially excluded from the protein surface, albeit to a lesser extent than expected. The authors point out the problems associated with choosing the right modelling parameters, in particular the water model (TIP3P) and calculation of the partial atomic charges of ectoine by *ab initio* methods in the gas phase. This may not adequately represent the real situation as indicated by seemingly excessive self-association of ectoine molecules (and concomittant lack of affinity for water) in the model. They come to the conclusion that in reality ectoine accumulates water molecules strongly and as a result has only restricted access to the protein surface. This conclusion is corroborated by others who also state that ectoine binds water strongly, at least in comparison to urea, resulting in an ordered hydration sphere [52]. It was also noticed that ectoine molecules are more strongly excluded from β-sheet and α-helix regions. In addition, they observed a significant elongation of water residence time in the protein interior and slowed down diffusion (50% smaller) around the protein backbone [53, 54]. It was concluded that slowdown of water diffusion around backbone amide protons must be the decisive factor in reducing the exchange rate of backbone amide protons. The influence of solutes (in this case glycine) on amide proton exchange rate had previously been demonstrated [55] using NMR techniques. The authors proposed a correlation between thermodynamic stabilization and decrease in exchange rate of slowly exchanging amide proton, i.e. those burried inside and requiring some degree of disruption to become solvent exposed. These findings corroborate the view of a more compact (less flexible) protein conformation in the presence of compatible solutes.

Differential Scanning Calorimetry (DSC)

DSC on reversible systems, often bovine ribonuclease A (RNase A), is used to retrieve thermodynamic data on protein stability. In the presence of compatible solutes an increase in melting temperature T_m is observed, which can be taken as a first indication for a stabilizing function [56, 57]. Experiments on a series of N-methylated solutes (i.e. betaine, dimethylglycine, sarcosine) revealed a concentrat-ion-dependent increase in melting temperature which was attenuated above a certain concentration (Fig. **3**). A detailed investigation comparing the stabilizing effect of betaine and hydroxyectoine on RNase A [58] revealed that both solutes increase the melting temperature in a linear fashion (approx. 3 K and 4 K per mol, respectively) up to a concentration of 3 M. At higher concentrations the T_m -raising ability of betaine (and other N-methylated compounds) gradually leveled off.

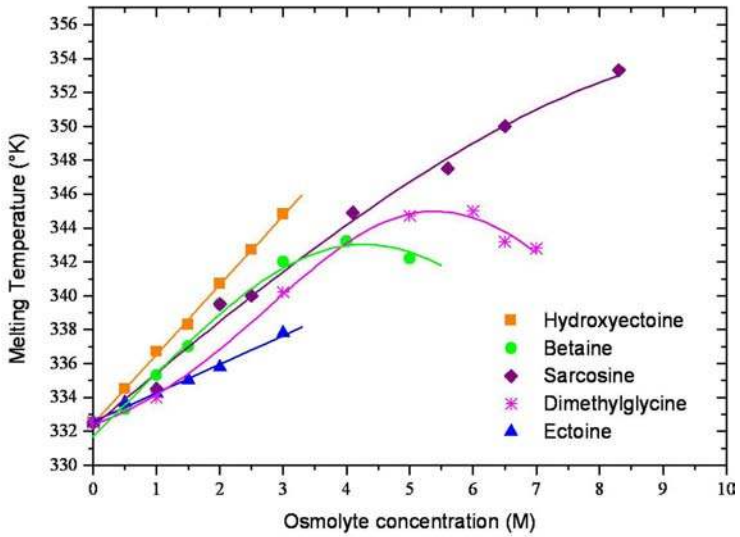

Fig. (3). Concentration dependent increase in melting temperature of RNase A. Calorimetrically determined increase in melting temperature (T_m) of RNase A is plotted against osmolyte concentration. Typical compatible solutes display a temperature-stabilizing effect in this model system, albeit to different degrees. It is worthy of note that solutes of extreme solubility (in particular betaine and dimethyl glycine) display partly reversed effects at very high concentrations. Data from Knapp *et al*. (1999) [58] and Voß (2002) [59] combined with normalized data on sarcosine and dimethylglycine from Santoro *et al*. (1992) [56].

Ectoine also displayed a linear increase in melting temperature, albeit only half as pronounced as that of hydroxyectoine [59].

Although ΔT_m can be taken as a first indication for the degree of stabilization by a particular solute [60], one must keep in mind that the thermodynamic stability curve of a protein is described by the Gibbs-Helmholtz equation based on four parameters (1).

$$\Delta G=\Delta H-T\Delta S+\Delta Cp[(T-T_m) -T \times \ln(T/T_m)] \quad (1)$$

The shape of this curve $\Delta G(T)$ is governed by ΔH, ΔS and ΔCp (heat capacity), of which the latter determines the curvature of the stability function. At the melting temperature, when $T = T_m$, both ΔG and the heat capacity term become zero. Therefore T_m can be decribed as $T_m = \Delta H_m/\Delta S_m$. From this relation it is obvious that an increase in ΔH or a decrease in ΔS will enhance the temperature of denaturation (T_m). If both parameters go in the same direction, the effect of one is partially counteracted by the other.

A particular solute with a pronounced effect on the heat capacity change of the system (e.g. > 25% increase) will influence the shape of the stability curve to such an extent that the protein may become less stable at physiological temperature even though the T_m value may be increased [57, 58]. Therefore, only concise determination of the thermodynamic parameters ΔH, ΔS and ΔCp will eventually reveal how the stability curve of the protein is influenced by the presence of compatible solutes and whether its shape/curvature is changed. In a pioneering investigation comparing the influence of glycine betaine and hydroxyectoine on RNase A thermodynamic parameters [58], it was demonstrated that the increase in T_m was caused by elevating the Gibbs free energy (stability) curve, with a $\Delta\Delta G^{298}$ of 4.1 and 3.1 kJ/mol (equivalent to a 14% and 10% increase) for 1 M solution of glycine betaine and hydroxyectoine, respectively [58]. These results have subsequently been supplemented by others for trehalose and the charged compatible solute potassium mannosylglycerate [61] with observed $\Delta\Delta G^{333}$ (close to Tm) of 2.3 kJ/mol for trehalose and 3.0 kJ/mol for mannnosylglycerate, both at 0.5 M concentration. Although the results are similar (elevation of the stability curve) the underlying thermodynamic factors seem to be different. Whereas in the case of hydroxyectoine and glycine betaine, a positive $\Delta\Delta G$ is apparently caused by the combined effect of changes in all three parameters (ΔH, ΔS and ΔCp, increasing by 17%, 15% and 27%, respectively), the presence of trehalose apparentyl did not significantly change ΔCp (the increase in ΔH overcompensating a concomittant increase in ΔS) and stabilization by mannosylglycerate resulted mainly from a large decrease in ΔS (overcompensating a concomittant decrease in ΔH) [61]. Recently TMAO, the most powerful stabilizer according to the transfer free energy model, has also been included [62]. It was shown that this solute provides a $\Delta\Delta G^{298}$ of 6.9 kJ/mol (equivalent to a 16% increase) at 1 M concentration.

Subsequent DSC studies on α-chymotrypsin revealed simliarities between N-methylated compounds (TMAO, betaine and sarcosine) and stabilizing disaccharides (sucrose, trehalose) on the one hand (ΔH up and ΔCp down) in contrast to typical denaturants like urea and guanidinium chloride (ΔH down and ΔCp up) [46, 63, 64]. In all cases the authors were able to demonstrate that naturally occurring osmolytes, by virtue of opposite thermodynamic parameters, strongly counteract the destabilizing effect of denaturants.

Although one must accept possible target-specific differences, the message from calorimetric investigations available so far is that the stability curve of proteins is usually raised in the presence of compatible solutes in thermodynamic terms. As a consequence compatible solutes (including ectoines) are able to compensate the destablizing

effect of heat, typical denaturants like urea and very likely other destructive factors.

CONCLUSIONS

Wiggins [35, 36] pointed out that the hydrophobic methyl groups in connection with a positive charge may be an essential prerequisite for a good compatible solute, because it can help to alter water structure while at the same time hydrophobic aggregation is prevented because of the repelling charge. This principle would apply to both, betaines and ectoines, the latter with its positive charge distributed between the two nitrogens. In view of the finding by Street *et al*. [40] it can similarly be concluded that methyl groups on positively charged atoms increase hydrophobicity (reducing the fractional polar surface area) while at the same time maintaining solubility. All the above observations can be condensed to the following biophysical principles for stabilizing solutes: a) a strong (kosmotropic) interaction with water, b) subsequent exclusion from protein surface, c) decrease of solubility of peptide backbone and d) strengthening of intramolecular hydrogen bonds (secondary structures).

Even though these basic principles of protein stabilization appear to be the same with different compatible solutes, it cannot be ignored that the combination of protein and corresponding stabilizing solute seems to matter and that some solutes excel as stabilizers for particular stress situations. Whereas, for example, glycine betaine is very suitable for heat protection of lactate-dehydrogenase (LDH), it conveys little protection against freeze-thawing, while TMAO excels [65, 66]. Hydroxyectoine, on the other hand, is an excellent heat protectant for both LDH and ribonuclease A (RNase A) [58, 65], while ectoine, a good protectant for LDH, has a markedly smaller effect on the heat stability of RNase A [59]. This heat protection of hydroxyectoine is also reflected at the whole-cell level [67]. The molecular basis of this function is however not yet understood. Although additivity in the backbone transfer model of hydration has been demonstrated [42, 68], it comes without saying that proteins are not all alike and that side chains do matter to some extent [38]. As shown for betaine-type solutes and proline [69] transfer free energy (ΔG_{tr}) from water to osmolyte solution can be negative for charged and aromatic amino acid side chains. This effect is usually overcompensated by the large positive value for the backbone. A specific affinity of compatible solutes towards certain amino acid side chains is also clearly reflected in the binding pockets of the ectoine binding proteins EhuB, TeaA and UehA of *Sinorhizobium meliloti, Halomonas elongata* and *Silicibacter pomeroyi*, respectively [70-72]. A protein-specific influence based on its amino acid composition is therefore hardly surprising. First attempts are now undertaken to compute a protein´s overall thermodynamic stability from its chain length and number of charged residues, which convey a destabilizing effect in the presence of osmolytes [73].

From what is known so far about ectoine-type solutes, it can be concluded that ectoine is a versatile stabilizing compound which seems to follow the principles outlined for other compatible solutes, in particular N-methylated compounds like TMAO, betaine, sarcosine etc.

There are, however, distinctive properties when compared to other compatible solutes (e.g. betaines). In contrast to betaine-type compatible solutes, ectoine is a strong UV-absorber and, so far, the only compatible solute which has successfully been introduced as in skin-care, skin-protection and health care products like eye drops, nasal sprays and dermatological creams with mitigating effects on inflammation. In addition, its preventive function on protein aggregation and distinct preserving power for damaged tissue (surgery) may open up further medical applications as outlined in the chapter Products.

Concerning the interaction with other biomolecules, as for example lipids of the cytoplasmic membrane, research on native fluid systems (as apposed to dry stabilization of lipsomes) is still at the beginning. Experimental evidence of the Galla group using the Langmuir film-balance technique and epifluorescence microscopy revealed striking effects of ectoines on the fluid-rigid domain distribution. The presence of ectoines evoked smaller and more numerous domains, thus expanding and fluidizing lipid monolayers [74]. Therefore, ectoines appear to have an effect on the line tension reducing the interfacial energy at the edge of the domains and on the mobility of the head groups. This effect was shown to be opposite to that of urea [52], which suggests that the counteractive function towards denaturants also applies to the fluid membrane system. Membrane interaction studies with ectoines were subsequently extended to the domain structure of artificial lung surfactant films, where their positive effect on biophysical compression/ expansion behaviour was confirmed [75]. In addition, infrared reflection-absorption spectroscopy revealed an increased tendency of hydroxyectoine at higher concentrat-ion (100 mM) to interact with phospholipid headgroups [75]. As N-methylated compatible solutes (TMAO, glycine betaine etc.) have so far not been included in such fluid membrane studies, it is not possible to conclude whether the observed effect on membranes is typical for ectoines or a general feature of compatible solutes.

PRODUCER STRAINS AND FERMENTATION PROCESSES

Ectoine Biosynthesis – Genes, Enzymes, and Regulation

Ectoine is produced by industry in a biotechnological process using the halophilic γ-proteobacterium *H. elongata*. *H. elongata* was isolated from a solar salt facility on Bonaire, Netherlands Antilles [76]. *H. elongata* is a moderately halophile having a genome that consists of a single chromosome (4 061 296 bp) and carries a total of 3473 protein-coding genes [11]. Its growth optimum is at salt concentrations between 3% and 6% (wt/vol) NaCl but *H. elongata* can still grow in minimal medium containing more than 20% salt [77]. An important osmoregulatory mechanism that allows for such a broad salt tolerance is the ability of *H. elongata* to synthesize the compatible solute ectoine [9]. Ectoine is synthesized from aspartate-semialdehyde, the central intermediate in the synthesis of amino acids belonging to the aspartate family (Fig. **4**). Ectoine formation comprises three enzymatic steps [78-81].

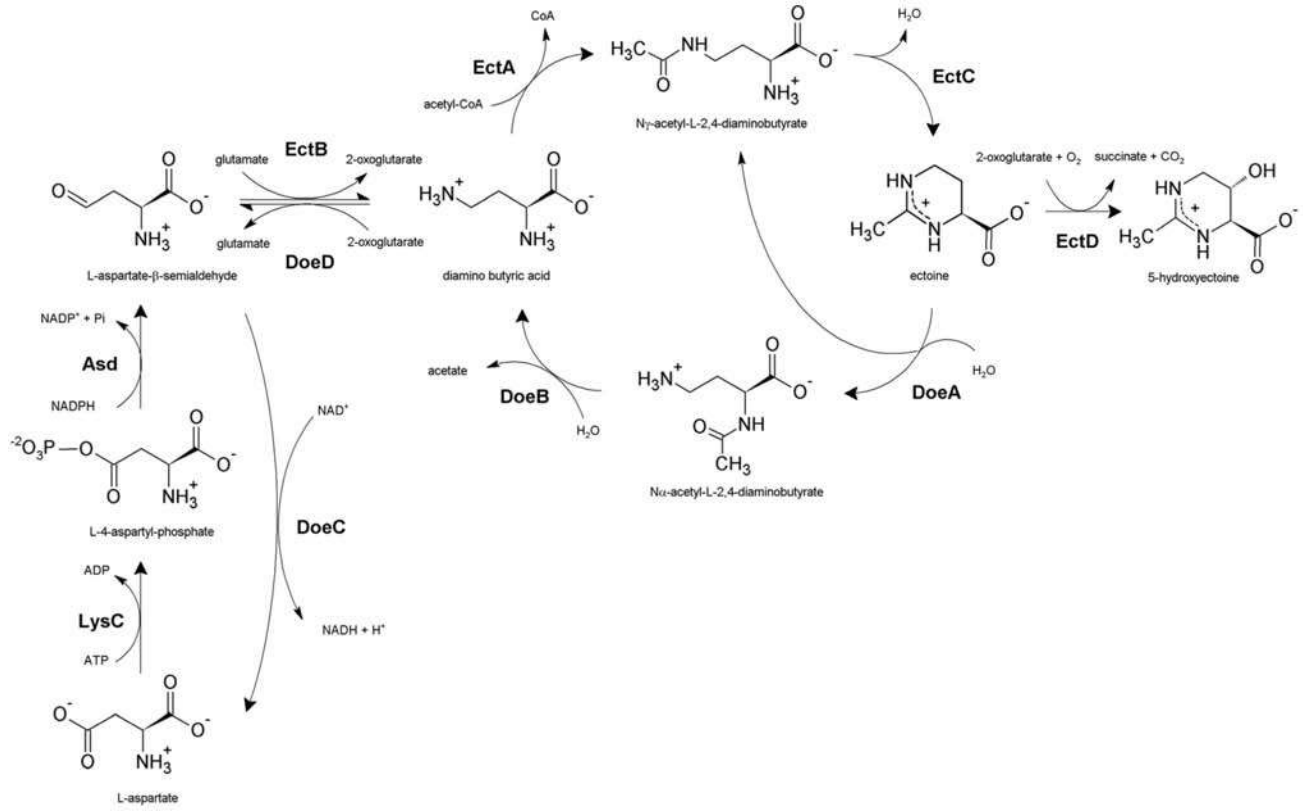

Fig. (4). Pathways of synthesis and catabolism of the compatible solutes ectoine and hydroxyectoine in *H. elongata*. The depicted ectoine biosynthesis and degradation pathway is according to studies previously published (11, 78, 81). LysC: aspartate kinase; Asd: aspartate-semialdehyde-dehydrogenase; EctB: L-2,4-diaminobutyric acid transaminase; EctA: L-2,4-diaminobutyric acid Nγ -acetyltransferase; EctC: ectoine synthase; EctD: ectoine hydroxylase; DoeA: ectoine hydrolase; DoeB: Nα-acetyl-L-2,4-diaminobutyric acid deacetylase; DoeD: L-2,4-diaminobutyric acid transaminase; DoeC: aspartate-semialdehyde dehydrogenase.

First, aspartate-semialdehyde is transaminated to 2,4-diaminobutyric acid (DABA) with glutamate as amino-group donor. The transamination is catalyzed by DABA transaminase EctB. EctB is a 421-residue protein with a molecular mass of 46.1 kDa, which requires K^+ for its transaminase activity and for protein stability. Gel filtration experiments with purified protein from *H. elongata* indicate that the DABA aminotransferase EctB might form a homohexamer in the native state.

Then, an acetyl group is transferred to DABA from acetyl-CoA by DABA-Nγ-acetyltransferase EctA in order to synthesize Nγ-acetyl-L-2,4-diaminobutyric acid. EctA is a 192-residue protein with a calculated molecular mass of 21.2 kDa.

Finally, ectoine synthase EctC catalyzes the cyclic condensation of Nγ-acetyl-2,4-diaminobutyric acid, which leads to the formation of ectoine. EctC is a 137-residue protein with a calculated molecular weight of 15.5 kDa and a pI value of 4.9. The EctC protein belongs to the enzyme family of carbon-oxygen lyases. *In vitro* experiments with purified EctC revealed that ectoine-synthase activity and affinity to its substrate are strongly affected by NaCl.

Under certain stress conditions (e.g. elevated temperatures) *H. elongata* converts some of the ectoine to 5-hydroxyectoine [82] by ectoine hydroxylase (EctD). The ectoine hydroxylase EctD consists of 332 amino acids and has a molecular weight of 37.4 kDa. The EctD protein is a member of an oxygenase subfamily within the non-heme-containing, iron (II)- and α -ketoglutarate-dependent dioxygenase superfamily. Ectoine hydroxylase was shown to catalyze the direct hydroxylation of ectoine to 5-hydroxyectoine [83].

The genes for ectoine biosynthesis *ectABC* (Helo_2588, Helo_2589, Helo_2590) are clustered together and can be found only 5 kb from the termination point of chromosome replication [11]. The *ectD* gene (Helo_4008) encoding the hydroxylase for hydroxyectoine synthesis is located apart from the *ectABC* cluster. Recently, Schwibbert and co-workers mapped the transcriptional initiation sites of *ectABC* and found two promoters in front of *ectA* and one upstream of *ectC* [11]. Upstream of *ectA* a putative σ^{70} promoter and an osmotically inducible σ^{38} promoter [84] were found, while in front of *ectC* a σ^{54}-controlled promoter is located. σ^{54}-controlled promoters are often involved in transcription of nitrogen-regulated genes [85, 86]. The transcriptional regulation of *ectABC* by an osmoregulated σ^{38} promoter and a σ^{54} promoter is in agreement with physiological observations made with other bacteria, such as *Corynebacterium glutamicum* and *Halorhodospira* (formerly *Ectothiorhodospira*) *halochloris*. In these organisms, it was shown that synthesis of the compatible solutes proline and glycine betaine, respectively, is not only determined by salinity but also by nitrogen supply [87, 88].

Ectoine biosynthesis is regulated at the level of transcription and enzyme activity [89]. Transcriptome analysis of *H. elongata* revealed an increase of mRNA from *ectA*, *ectB*, and *ectC* with increasing sodium chloride concentration from 0.6% (0.1 M) to 12% (2 M) NaCl (Kunte, unpublished results). Similar results were obtained from proteome analysis that showed an increase in EctABC protein at elevated salinities. Contribution of expression and enzyme activity of EctABC to osmoregulated ectoine synthesis was investigated with cells of *H. elongata* that have been treated with chloramphenicol (Cm) to abolish protein biosynthesis [89]. Cultures of *H. elongata* with and without Cm growing at 1% NaCl (0.17 M) and 4% NaCl (0.68 M) were exposed to an osmotic upshift. The salt concentration of the cultures growing at 1% NaCl was increased to 4% NaCl and in the cultures growing at 4% NaCl to 8% NaCl. The Cm treated cells grown at a low salt concentration of 1% NaCl failed to synthesize sufficient ectoine in response to the salt shock that raised the salinity to 4% NaCl. This observation indicates that at low salinities regulation of ectoine synthesis predominantly relies on expression. Contrary, cells adapted to an elevated salinity of 4% NaCl synthesized as much ectoine as the control cells without Cm after osmotic shock. Apparently, the EctABC proteins are osmoregulated enzymes that can react (directly or indirectly) to changes in osmolarity. These *in vivo* findings are supported by *in vitro* experiments carried out with purified EctABC enzymes. The activity of all three enzymes is strongly affected by increasing potassium and sodium salts [80].

Ectoine synthesis is not only affected by osmolarity but also by the presence of compatible solute such as glycine betaine transported to the cytoplasm *via* osmoregulated transporters. For energetic reasons, glycine betaine accumulation by transport or synthesis from choline is preferred over *de novo* synthesis of ectoine [90, 91]. Whether uptake of external compatible solutes regulates ectoine synthesis at the level of enzyme activity and/or at the level of transcription of *ectABC* has still to be resolved (see also below *The "leaky" mutant procedure*).

The "Bacterial Milking" Procedure

Ectoine can easily be synthesized chemically [18, 19]. However, large-scale chemical synthesis of ectoine is not competitive with biotechnological production because of the need for high prized precursors such as diaminobutyric acid. The first process that had been developed for the bio-technical production of ectoine was the so-called *bacterial milking* procedure [92]. Ectoine has a maximum solubility of 6.5 mol/kg water at 25 °C [93] and is accumulated up to molar concentration inside the cytoplasm. Halophilic bacteria such as *H. elongata* thriving in a high saline environment have to be attuned to a sudden decline in salinity caused by rainfall or flooding. In this situation the cell has to cope with a cytoplasm that has a significantly higher osmolarity (or lower chemical potential) than the surrounding environment causing a sudden influx of water (since water is freely permeable across the cell membrane). In order to avoid bursting, the cell has to release ectoine (and other compatible solutes) from the cytoplasm, probably through mechano-sensitive channels, of which *H. elongata* possesses three MscS and one MscK.

H. elongata combines several features making it a reliable industrial producer strain: it grows robustly on a wide variety of substrates it is safe as also indicated by its use in food processing [94] and can achieve high cell densities (>40 g dry weight/l corresponding to >10 g/l ectoine). The bacterial milking process exploits the ability of *H. elongata* to release ectoine in response to dilution stress to the medium. Cells of *H. elongata* are grown in a fed-batch fermentation process at a salinity of 10% NaCl (1.7 M) until a high cell density has been reached. Then, an osmotic down shock from 10% to 2% NaCl is applied. As a result, approximately 80% of the cytoplasmic ectoine is released to the culture medium. The excreted ectoine is then recovered from the medium by cross-flow filtration and further purified by cation exchange chromatography and crystallization. After harvesting, the filtered cell mass is reused and brought back into high saline (10% NaCl) culture medium for another round of ectoine synthesis. Within 10 hours the bacterial milking can be repeated. A significant increase in productivity could be achieved by replacing the fed-batch process with a continuous fermentation [95].

The "Leaky" Mutant Procedure (Exploiting a Mutant which Excretes Ectoine into the Medium)

Microorganisms do not rely entirely on *de novo* synthesis of compatible solutes. They are able to take up compatible solutes or precursors from the surrounding environment, if available. To allow for this uptake, microorganisms must be equipped with a set of specific transporters functioning at high osmolarity and high ionic strength, conditions that often inhibit transporters for nutrient uptake. These transporters facilitate a far more economical accumulation of compatible solutes compared to *de novo* synthesis [90]. *H. elongata* is equipped with a set of different compatible solute transporter [91] but only one transporter facilitates the (osmoregulated) uptake of ectoine namely the tripartite ATP-independent periplasmic transporter (TRAP-T) [96, 97] TeaABC [98]. TRAP-T are wide spread in organisms from the bacteria and archaea domain. Unlike the ABC transporters that are fuelled by ATP hydrolysis, TRAP-T are secondary transporters. Reconstitution experiments with the TRAP-T SiaPQM revealed that Na^+ is the coupling ion for transport. This finding is supported by the fact that TRAP-T can be found predominantly in marine bacteria, which prefer to utilize Na^+ as a counter ion due to its abundance. The actual transporter TeaABC consists of three proteins, a large transmembrane protein (TeaC, Helo_4276), a small transmembrane protein (TeaB, Helo_4275), a periplasmic substrate-binding protein (SBP; TeaA, Helo_4274) with a high affinity for ectoine [71, 99]. In addition, a regulatory ATP-binding protein TeaD (Helo_4277) is influencing the activity of TeaABC. The larger transmembrane protein is thought to catalyze the actual transport reaction and is responsible for energy coupling most likely through the sodium motive force. The small membrane protein is not involved directly in the transport reaction and its exact function is still unknown. Since the small subunit is almost always encoded upstream of the gene for the large subunit, a chaperone-like function in the folding of the large membrane protein is suggested.

Another hypothesis brought forward is that the small subunit might be needed to interact with the SBP for transport. TeaD is an ATP-binding protein and functions as a negative regulator of the TeaABC transporter [100]. However, the exact role of TeaD in regulating TeaABC is still unknown.

Because osmoregulated transporters such as TeaABCD are exposed to both the high salt environment and the cytoplasm, it was hypothesized that these systems would also function as sensors for osmotic changes. This was shown to be the case for different compatible solute transporters. In addition, compatible solute transporters must be integrated (directly or indirectly) in the regulation of the cell's compatible solute synthesis, because the uptake of external solutes results in an immediate decrease in the concentration of compatible solutes synthesized by the cell [91]. In some bacteria transporters for compatible solutes are not restricted to the accumulation of external osmoprotectants, but are also functioning as a salvage system for compatible solutes leaking out of the cell [101]. That was shown to be true for *H. elongata* [98, 102]. Mutation of the ectoine transporter TeaABC (Δ*teaC*) resulted in a mutant excreting ectoine to the medium (Fig. **5**).

Quantifying the excreted ectoine and the cytoplasmic ectoine revealed that the TeaABCD transporter mutant is both an ectoine leaky mutant and an overproducer for ectoine [103]. Why ectoine is leaking out the cell when the specific transporter for ectoine is mutated is not quite understood yet.

Similar observations have been recorded for the cyanobacterium *Synechocystis* sp. strain PCC 6803, which synthesizes glucosylglycerol as its main compatible solute [101, 104], and *Bacillus subtilis*, which synthesizes proline as compatible solute [105]. The mutation of *ggtA,* a gene encoding a subunit of an ABC transport system mediating the uptake of glucosylglycerol in *Synechocystis* sp. created a mutant leaky for glucosylglycerol, while the mutation of the osmoregulated proline transporter *opuE* in *B. subtilis* created a leaky mutant for proline. However, the mutation of the transporter in *B. subtilis* did not create an overproducing strain as observed for *H. elongata*, but quite contrary a mutant that synthesizes less proline than the parental strain (see also below).

However, there is evidence that the loss and the recovery of ectoine by the TeaABC transporter might be an elegant mechanism to regulate the cytoplasmic ectoine content in *H. elongata* [106]. As mentioned above, compatible solute transporters have to be linked to ectoine synthesis in order to shut down ectoine synthesis when external solutes are taken up. The same must be true for TeaABCD in order to regulate ectoine synthesis when external ectoine is transported into the cytoplasm. This implies that any ectoine, regardless of its origin (even ectoine lost from the cell) will have this negative regulatory effect on the synthesis of ectoine. It is therefore possible that ectoine transport could serve as a signal for the regulation of ectoine synthesis. Increasing the ectoine concentration by *de novo* synthesis will lead to water influx and an increase in turgor pressure of salt-stressed cells. Assuming that the efflux of ectoine *via* an export channel is triggered by a signal such as membrane stretch, ectoine will be released to the periplasm if the turgor pressure reaches a certain threshold. Transport of exported ectoine back into the cytoplasm by the activated TeaABCD system will down regulate ectoine synthesis (Fig. **6**). The proposed regulation mechanism would allow for an oscillation of the cytoplasmic ectoine level closely above and below the threshold needed to open the solute-specific export channels. As a consequence, in cells with a functional TeaABC transporter ectoine will be cycled between the outside (periplasm) and the cytoplasm.

The described model is supported by the findings made with other bacteria. For *Corynebacteria glutamicum* it was shown that the uptake of the compatible solute glycine betaine by transporter BetP and its concomitant export *via* the stretch sensitive channel MscCG might be involved in regulation of the steady state concentration of compatible solutes in the cytoplasm which are accumulated in response

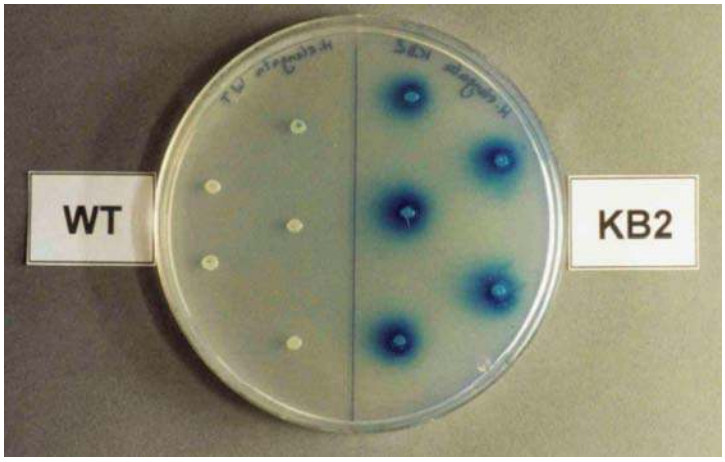

Fig. (5). Ectoine excreting mutant *H. elongata* KB2 (Δ*teaC*) and wild type (WT) *H. elongata* DSM 2581^T on an assay agar for the detection of ectoine. Ectoine excretion is indicated by the blue halo around the mutant KB2. The detection assay employs *Escherichia coli* K-12 as an indicator organism for ectoine. An overnight culture of *E. coli* (100 µl) was spread onto minimal medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 900mM NaCl, which inhibits the growth of *E. coli* in the absence of external compatible solutes. *H. elongata* WT and excretion strain *H. elongata* KB2 were transferred onto the assay plate and incubated for 48 hrs at 37 °C. Growth of *E. coli* next to ectoine-excreting colonies is indicated by X-Gal, which is converted into a blue dye by the β-galactosidase activity of growing *E. coli* cells.

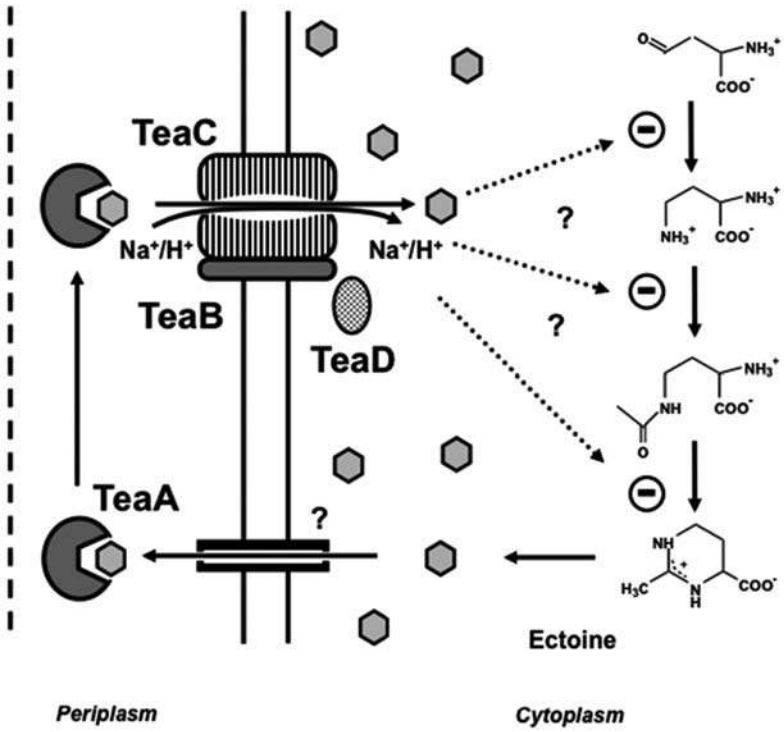

Fig. (6). Model describing the regulation of the cell's ectoine synthesis by osmoregulated transporter TeaABCD. Ectoine is synthesized and exported to the periplasm. Periplasmic ectoine is taken up by the specific osmoregulated transport system TeaABCD. Uptake of ectoine down regulates ectoine *de novo* synthesis. The model is based on the facts that (i) bacteria like *H. elongata* will lose their synthesized compatible solutes such as ectoine to the medium if the specific transporters are inactivated, and that (ii) compatible solute transport into the cell shuts down endogenous solute synthesis.

to hyperosmotic stress [107]. In this context, it is important to mention that for the above described *opuE* mutant of *B. subtilis* the involvement of mechanosensitive channels in the release of the compatible solute proline could be ruled out [108]. However, it is also important to point out the differences in the transport mutants of *B. subtilis* and *H. elongata*. The intracellular proline content in the *opuE* mutant from *B. subtilis* is considerably lower than that of its parental strain and as a consequence the mutant is salt sensitive. In contrast, *teaABC* mutants of *H. elongata* have the same intracellular ectoine content and display the same salt tolerance as the wild type cells [98]. As explained before, *teaABC* mutants of *H. elongata* are overproducing mutants, which indicates are regulatory connection between release and uptake of ectoine. These differences suggest a different osmoregulatory mechanism in *B. subtilis* compared to *H. elongata* in controlling the compatible solute content.

Employing the ectoine over-producing "peeing" mutant of *H. elongata* for large-scale industrial production of ectoine offers a number of advantages over the previously used bacterial milking procedure (Fig. **7**). Here, the production of ectoine is completely uncoupled from intracellular accumulation of ectoine, therefore the yield in g/g on carbon source is substantially increased. This means that also the product titer is no longer limited by the biomass density that can be achieved. The process can be performed in continuous culture, but also in the simpler fed-batch or batch mode with the advantages of increased robustness and straightforward scalability. Also, the amount of salt in the process can be reduced to the level needed for the activation of ectoine biosynthesis enzymes, decreasing the salt burden that needs to be separated in the downstream processing. Furthermore, the number of downstream processing operations can be reduced, since no more osmotic down shock and second filtration step is needed (Fig. **7**). Altogether, the "leaky" production strain allows for a process more similar to the highly efficient fermentation processes employed for the manufacture of amino acids like lysine.

Blocking Ectoine Degradation – The "Super-Leaky" Mutant

Ectoine is accumulated as an osmolyte in *H. elongata*. However, ectoine can also be used as both a carbon and nitrogen source and when offered as a nutrient, ectoine still serves as compatible solute. In a combined effort of research and industry the genome of *H. elongata* was sequenced. Genome analysis and mutagenesis experiments let to the discovery of the pathway for ectoine degradation [11]. The *d*egradation *o*f *e*ctoine (*doe*) to aspartate comprises four steps and proceeds *via* hydrolysis of ectoine to Nα-acetyl-L-2,4-diaminobutyric acid, followed by deacetylation to diaminobutyric acid. In *H. elongata,* diaminobutyric acid can either flow off to aspartate or re-enter the ectoine synthesis pathway, forming a cycle of ectoine synthesis and degradation (Fig. **4**).

The ectoine hydrolase DoeA, which catalyzes the first step in ectoine degradation, is a 399 aa protein (44.9 kDa, pI 5.0) that belongs to the peptidase-M24 family. Within that family, DoeA is similar to creatinase (creatine amidino-

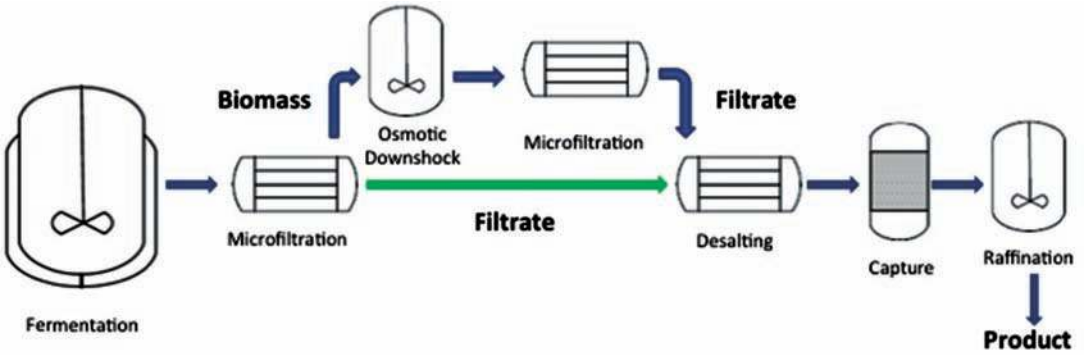

Fig. (7). Schematic view of the *bacterial milking* and *leaky mutant* bioprocesses for ectoine. In the bacterial milking process (blue arrows), the biomass is concentrated by microfiltration, the concentrate is submitted to an osmotic down shock by dilution with water. The released ectoine is separated from the biomass by a second microfiltration. The further downstream processing includes an electro-dialysis for desalting, a capture to a chromatographic resin to remove impurities from the fermentation broth (e.g. color), and crystallization and drying as final purification steps. For the leaky strain, the downstream processing can be simplified and the first microfiltration is directly followed by the desalting step (green arrow).

hydrolase), which catalyzes the hydrolysis of creatine to sarcosine and urea. The essential product of ectoine hydrolysis catalyzed by DoeA is Nα-acetyl-L-2,4-diaminobutyric acid (Nα-Ac-DABA), which is the substrate for the subsequent catabolic enzyme DoeB. In recombinant *E. coli* expressing doeA also Nγ-Ac-DABA was detectable. Whether DoeA catalyzes the formation of both isoforms (Nα-Ac-DABA and Nγ-Ac-DABA) or if the cleavage produces only one isoform that is subsequently converted into the corresponding isomer by an acetyltransferase is not quite clear yet. The Nα-acetyl-L-2,4-diaminobutyric acid deacetylase DoeB is 342 aa protein (36.6 kDa, pI 4.6) and closely related to proteins of the succinyl-glutamate desuccinylase/aspartoacylase subfamily, which are part of the M14 family of metallocarboxypeptidases. DoeB deacetylates Nα-Ac-DABA to L-2,4-diaminobutyric acid (DABA), which can serve as substrate for the catabolic transaminase reaction DoeD (Fig. **4**). DoeD is a 469 aa enzyme (50.8 kDa, pI 5.6) that belongs to the PLP-dependent aspartate aminotransferase superfamily. DoeD catalyzes the formation of aspartate-β-semialdehyde, which finally can be converted to aspartate by dehydrogenase DoeC (493 aa, 53.1 kDa, pI 4.8).

The genes encoding the ectoine degradation pathway are located together on the chromosome of *H. elongata*. The genes *doeA* and *doeB* are organized as an operon together with a third gene called *doeX.* The *doeCD* components are located adjacent to *doeABX* but are not part of the *doeABX* operon. Promoter-mapping analysis revealed the presence of a σ^{70}-dependent promoter in front of the *doeABX* operon. The *doeX* gene encodes for DNA-binding protein belonging to the Asn/Lrp family of DNA-binding proteins and binds to a 46 nt sequence located directly upstream to the *doeA* start codon. The regulatory impact of DoeX on the *doeAB* expression is still unknown.

As indicated above, DABA can be degraded to aspartate or re-enter the ectoine synthesis pathway *via* EctA and EctC. In this way degradation and synthesis of ectoine are directly connected forming a cycle that is powered by the acetylation reaction catalyzed by EctA. However, this cycle appears to be futile, in which ectoine can be synthesized and degraded simultaneously resulting in a net conversion of acetyl-CoA into acetate. Acetate can be reconverted to acetyl CoA by acetate : CoA ligase (AMP-forming; EC 6.2.1.1). Therefore, if an ectoine synthesis and degradation cycle were active, it would result in an increased cost of two ATPs per turn for ectoine synthesis. Kunte and co-workers [11] showed that such a cycle is an elegant mechanism to control the speed of change in internal ectoine concentration as response to external changes in osmolarity. It is known that the turnover time of a metabolite, defined as the ratio between its concentration and the flux through it in the steady state, is a good indicator of the timescale of its transient responses [109]. Metabolites with a high turnover tend to complete transitions faster than those with a low turnover. Thus, by keeping a flux through the synthesis/degradation cycle, the cell can achieve fast changes in ectoine levels to quickly respond to changes in external osmolality. Based on this model, a disruption of the ectoine degradation pathway should lead to a lower ATP load for cells synthesizing ectoine and at the same time in higher ectoine productivity. In fact, deletion of the *doeA* gene in an ectoine excretion mutant (KB2.11; Δ*teaABC*) resulted a strain of *H. elongata* with a three times higher productivity compared to the leaky mutant (unpublished data). This new strain with a blocked ectoine degradation pathway and thereby increased ectoine excretion offers new options for the large-scale production of ectoine.

PRODUCTS

The first commercial use of Ectoine was as a skin care ingredient [110, 111] and this application still plays a major role, especially in sun protection and anti-aging products where ectoine is widely used [23, 112, 113]. Recently, the use of ectoine in health care products has become of increasing importance. Starting with the work of Buenger and Driller [114] and Grether-Beck *et al*. [115], which showed that ectoine is able to inhibit the early UV-A radiation-induced ceramide signaling response in human keratinocytes, the mitigating effect of ectoine on inflammatory conditions of human skin was examined further. Pre-treatment of keratinocytes with ectoine reduced the number of sunburn cells, prevented the decline in Langerhans cells [116] and decreased UV-induced DNA single strand breaks [117]. *In vitro* studies on ectoine´s

Fig. (8). Ectoine-based medical device products. Ectoine-based health products on the market encompass allergy nasal sprays and allergy eye drops, eye drops and nasal sprays for the treatment of dry eyes and dry nose, products for the treatment of cough and cold and dermatological crèmes for the treatment of atopic dermatitis and other inflammatory skin diseases.

beneficial effects for human skin are confirmed by clinical trials on skin aging [118]. Here it could be demonstrated that a formulation with already good skin care properties could be significantly improved by the addition of 2% ectoine, which led to superior skin hydration, skin elasticity and skin surface structure. The mitigating effect of ectoine was not only observed in skin but also in inflammatory conditions of other epithelia. For lung epithelia, it was shown that ectoine protects against nanoparticle-induced airway inflammation [13]. For nasal and eye epithelia in allergic conjunctivitis, ectoine containing nasal spray and eye drop products relieved effectively the hallmark symptoms of rhinoconjunctivitis with treatment effects similar to those of antihistamines, steroids and leukotriene modifiers, but with virtually no side effects [119].

Based on this, a wide range of Ectoine-based medical device products for the treatment of allergies (allergy nasal sprays and eye drops), skin inflammatory conditions like atopic dermatitis, the treatment of dry eye and dry nose and rhinosinusitis have been developed, successfully tested in clinical trial [120] and have entered the market (Fig. **8**). Due to the excellent safety profile in combination with clinically proven efficacy in the treatment of inflammatory conditions of epithelia, an even wider use of ectoine can be envisaged in the future. Potential future applications include the treatment of epithelial derived inflammatory diseases, especially nano-particle induced, lung inflammation [121], colitis [122] and tissue protection in ischemia [123, 124].

In addition, the effect of ectoine and related compatible solutes in preventing aggregation of amyloidogenic proteins by stabilizing the native state [15, 45, 116, 117] may serve as a promising starting point for the development of amyloid-inhibiting novel compounds for the treatment of neurode-generative disorders such as Alzheimer's, Parkinson´s or Huntington´s as well as prion-related diseases [15, 47, 125-128].

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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