Review

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The ups and downs of ectoine: structural enzymology of a major microbial stress protectant and versatile nutrient

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Abstract: Ectoine and its derivative 5-hydroxyectoine are compatible solutes and chemical chaperones widely synthesized by *Bacteria* and some *Archaea* as cytoprotectants during osmotic stress and high- or low-growth temperature extremes. The function-preserving attributes of ectoines led to numerous biotechnological and biomedical applications and fostered the development of an industrial scale production process. Synthesis of ectoines requires the expenditure of considerable energetic and biosynthetic resources. Hence, microorganisms have developed ways to exploit ectoines as nutrients when they are no longer needed as stress protectants. Here, we summarize our current knowledge on the phylogenomic distribution of ectoine producing and consuming microorganisms. We emphasize the structural enzymology of the pathways underlying ectoine biosynthesis and consumption, an understanding that has been achieved only recently. The synthesis and degradation pathways critically differ in the isomeric form of the key metabolite *N*-acetyldiaminobutyric acid (ADABA). *γ*-ADABA serves as preferred substrate for the ectoine synthase, while the α-ADABA isomer is produced by the ectoine hydrolase as an intermediate in catabolism. It can serve as internal inducer for the genetic control of ectoine catabolic genes via the GabR/MocR-type regulator EnuR. Our review highlights the importance of structural enzymology to inspire the mechanistic understanding of metabolic networks at the biological scale.

Keywords: chemical chaperones; compatible solutes; enzymes; gene regulation; microbial physiology; osmotic stress; structural analysis.

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Introduction: managing water stress

Changes in the environmental osmolarity, a parameter to which most microorganisms are frequently exposed (Gunde-Cimerman et al. 2018; Kempf and Bremer 1998; Roeßler and Müller 2001), impinge on hydration of the cytoplasm, magnitude of turgor, growth and cellular survival (Bremer and Krämer 2019; Wood 2011). Microorganisms lack the ability to actively pump water across the cytoplasmic membrane to counteract water fluxes in or out of the cell that are triggered by increases or decreases in the

external osmolarity. Hence, as water passes along pre-existing osmotic gradients through the semi-permeable cytoplasmic membrane, bacterial cells have to counteract hypo- and hyper-osmotic challenges through an active, yet indirect, water-management to avoid dehydration under hyper-osmotic circumstances and cell rupture under hypo-osmotic conditions. Directing and scaling these water fluxes is key for the cellular osmostress response of microorganisms (Bremer and Krämer 2019; Wood 2011).

Upon sudden exposure to hypo-osmotic circumstances, microorganisms reduce the osmotic potential of their cytoplasm through the transient opening of mechanosensitive channels. Consequently, ions and metabolites are rapidly jettisoned, thereby curbing water influx, an undue increase in turgor and in extreme cases cell rupture is avoided (Booth 2014). Conversely, when microorganisms face hyper-osmotic surroundings, they initially accumulate potassium ions, and on a longer time scale, a particular class of physiologically compliant organic osmolytes, the compatible solutes (Brown 1976). This adjustment strategy to acute and sustained osmotic stress, often referred to as the "salt-out" response, aims at avoiding a long-lasting increase in the ionic strength of the cytoplasm to maintain the functionality of the cell's constituents and biochemical activities (Galinski and Trüper 1994; Gunde-Cimerman et al. 2018; Kempf and Bremer 1998). Collectively, the accumulation of compatible solutes prevents dehydration of the cytoplasm, an undue increase in molecular crowding, and a drop in turgor to non-physiologically adequate values (Bremer and Krämer 2019; van den Berg et al. 2017; Wood 2011) (Figure 1).

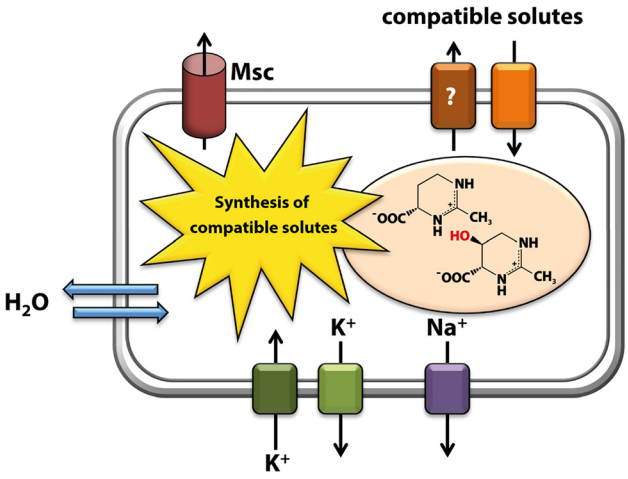

Figure 1: The core of the salt-out osmostress response. Shown are the systems that mediate cellular import of K^+ under acute osmotic stress as an immediate adjustment response and those that allow it extrusion once the cell begins with the import and/or synthesis of compatible solutes (e.g., ectoine and its derivative 5-hydroxyectoine) (Bremer and Krämer 2019; Wood 2011). Cells also seem to possess efflux systems for compatible solutes, probably a measure to fine-tune turgor when the cell elongates and doubles its volume prior to division (Czech et al. 2016; Vandrich et al. 2020). Cells maintain very low intracellular Na^+ concentration through dedicated export systems as this ion, in contrast to K^+, is cytotoxic (Danchin and Nikel 2019). Overall, cells that use the *salt-out* osmostress response, in contrast to those that use high concentrations of K^+/Cl^- to balance the osmotic gradient (the *salt-in* response) (Gunde-Cimerman et al. 2018), strive to prevent the development of a long-lasting high-ionic strength cytoplasm. The accumulation of compatible solutes is a key component of the acclimatization to both acute and sustained high osmolarity surroundings by microbial cells (Bremer and Krämer 2019; Wood 2011). The designation Msc represents MscS- and MscL-type mechanosensitive channels serving as safety valves for the rapid release of ions and organic solutes upon sudden osmotic down-shocks (Booth 2014).

Compatible solutes are operationally defined as highly water-soluble organic molecules that can be accumulated by both pro- and eukaryotic cells to exceedingly high intracellular levels without impairing the physiology and biochemistry of the cell (Brown 1976; Yancey 2005). The cytoprotective effects of compatible solutes are routinely interpreted in the framework of the 'preferential exclusion model' (Arakawa and Timasheff 1985; Bolen and Baskakov 2001), which proposes that these molecules are preferentially excluded from the protein backbone (Street et al. 2006; Capp et al. 2009; Cayley and Record 2003; Zaccai et al. 2016). This physico-chemical property of compatible solutes generates a thermodynamic driving force for proteins to minimize their exposed surface in order to keep the uneven distribution of compatible solutes in the cell water at a minimum. Hence, compatible solutes act against the denatured state and thereby promote the correct folding and appropriate hydration of proteins (Bolen and Baskakov 2001). Maintaining functionality of macromolecules through compatible solutes cannot only be observed *in vitro* for isolated proteins (Lippert and Galinski 1992), but most importantly also *in vivo* (Barth et al. 2000; Bourot et al. 2000; Caldas et al. 1999; Ignatova and Gierasch 2006; Stadmiller et al. 2017). These solutes also protect the functionality of other macromolecules (e.g., nucleic acids) and cellular compartments (e.g., membranes) (Hahn et al. 2017, 2020; Harishchandra et al. 2010, 2011; Herzog et al. 2019; Kurz 2008). Accordingly, compatible solutes can also be addressed as 'chemical chaperones' (Chattopadhyay et al. 2004; Diamant et al. 2001). The physico-chemical characteristics of compatible solutes and their high water-solubility make them ideally suited as osmostress protectants as they can be accumulated to high cytoplasmic pools, a process occurring in tune with the degree of the imposed osmotic stress onto the microbial cell (Czech et al. 2018b; Kuhlmann and Bremer 2002).

Microorganisms synthesize or import a great variety of compatible solutes (da Costa et al. 1998; Gunde-Cimerman et al. 2018; Kempf and Bremer 1998; Klähn and Hagemann 2011; Roeßler and Müller 2001). However, those that are synthesized by members of the *Bacteria* belong only to a restricted number of chemical classes: quaternary amines (e.g., glycine betaine), amino acids (e.g., L-proline), amino acid derivatives (e.g., ectoine), sugars (e.g., trehalose), and polyoles (e.g., glycerol, glucosylglycerol). Here, we focus on ectoine and its derivative 5-hydroxyectoine (Figure 1), cytoprotectants that are among the most widely synthesized compatible solutes in the microbial world (Czech et al. 2018a; da Costa et al. 1998; Galinski and Trüper 1994).

Ectoines: broadly synthesized and highly effective microbial cytoprotectants

The tetrahydropyrimidines ectoine [(4*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] and its derivative 5-hydroxyectoine [(4*S*,5*S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] stand out from other compatible solutes due to their excellent cytoprotective attributes. In addition, numerous biotechnological and biomedical applications for these compounds have been developed (Becker and Wittmann 2020; Czech et al. 2018a; Jorge et al. 2016; Kunte et al. 2014; Pastor et al. 2010). Ectoine was originally discovered by Galinski et al. (1985) in the halophilic phototrophic bacterium *Ectothiorhodospira halochloris* (Galinski et al. 1985; Schuh et al. 1985), followed by the discovery of its derivative 5-hydroxyectoine just a few years later in the soil bacterium *Streptomyces parvulus* (Inbar and Lapidot 1988). Ectoines are zwitter-ions in solutions of neutral pH. They are highly soluble in water [up to 4 M at 20 °C and up to 6 M at 4 °C (Schuh et al. 1985; Zaccai et al. 2016)], and affect the hydrogen bonding network of water on a local scale (Sahle et al. 2018). Molecular dynamics simulations and dielectric relaxation spectra predict strong hydrogen bonds formed between water and ectoine and 5-hydroxyectoine (i.e., seven and nine water molecules, respectively) (Eiberweiser et al. 2015; Smiatek et al. 2012).

Synthesis and import of ectoines contribute greatly to osmotic stress resistance in many microorganisms as their accumulation averts water loss and provides functional protection of cellular constituents and biosynthetic processes (Czech et al. 2018a; Galinski and Trüper 1994; Gunde-Cimerman et al. 2018; Kunte et al. 2014; Leon et al. 2018; Pastor et al. 2010; Reshetnikov et al. 2011a; Schwibbert et al. 2011). Moreover, ectoines can also serve as cytoprotectants for either cold- or heat-challenged cells (Bursy et al. 2008; Garcia-Estepa et al. 2006; Kuhlmann et al. 2008a, 2011; Malin and Lapidot 1996; Ma et al. 2017). However, the molecular underpinnings for their function as thermoprotectants are less well-understood; these probably depend on the chemical chaperone properties of ectoines. For instance, ectoines might stabilize a selected set of proteins crucial for temperature stress resistance or preserve vital biosynthetic processes (Bayles et al. 2000; Biran et al. 2018; Zhao et al. 2019). 5-Hydroxyectoine is also an excellent desiccation protectant (Manzanera et al. 2004), an attribute that probably depends on its enhanced ability to produce glasses in comparison with ectoine (Tanne et al. 2014).

Organisms that produce both ectoine and 5-hydroxyectoine often accumulate a mixture of these solutes, even though the ratio of the two ectoines can vary between species (Bursy et al. 2008; Czech et al. 2016; Pastor et al. 2010; Stöveken et al. 2011; Tao et al. 2016). Moreover, some microorganisms accumulate higher amounts of 5-hydroxectoine when they enter stationary growth phase (Saum and Müller 2008; Schiraldi et al. 2006; Seip et al. 2011; Tao et al. 2016). This observation points to superior stress-relieving properties of 5-hydroxyectoine for cells when nutrients become limiting, growth slows down, and cells face a multitude of new challenges (Hengge-Aronis 2002).

Phylogenomic distribution of ectoine-producing microorganisms among *Bacteria*

Although initially widely considered as rarely occurring microbial compatible solutes, the discovery of the ectoine (*ectABC*) (Louis and Galinski 1997) and 5-hydroxyectoine (*ectD*) biosynthetic genes (Bursy et al. 2007; Garcia-Estepa et al. 2006; Prabhu et al. 2004), coupled with an ever increasing availability of genome sequences, revealed the wide distribution of ectoines in members of the *Bacteria*. However, their occurrence in *Archaea* is restricted (Czech et al. 2018a; Kunte et al. 2014; Pastor et al. 2010; Widderich et al. 2016a) (Figure 2). Building on recent searches of the IMG/MER database of microbial genomes and metagenomic assemblies (Chen et al. 2019), and considering only fully sequenced bacterial and archaeal genomes (Czech et al. 2018a; Mais et al. 2020), the distribution of the ectoine biosynthetic genes in 8 850 fully sequenced genomes (8557 *Bacteria* and 293 *Archaea*) was assessed (Figure 2). In this dataset, 665 predicted ectoine producers originated from *Bacteria* while 11 representatives belong to

the *Archaea* (Supplementary Figure S1). This corresponds to an occurrence of ectoine biosynthetic genes in approximately 7.5% of all queried 8 850 fully sequenced microbial genomes. These numbers are for obvious reasons only fleeting snapshots. Given that incompletely sequenced microbial genomes and metagenomic sequences were not taken into account, it is highly likely that the number of microbial ectoine producers will considerably exceed the number derived from the inspection of only fully sequenced microbial genomes (Mais et al. 2020). Studies on the taxonomic affiliation of ectoine-synthesizing microorganisms revealed their presence in 10 bacterial and two archaeal phyla (Figure 2). This analysis also illustrates that the *ect* genes are not evenly distributed among members of the *Bacteria*: 38% are present in Actinobacteria, 6% are found in Bacilli, and 53% occur collectively in the *α*-, *β*-, and *γ*-Proteobacteria.

While ectoine producers are often referred to in the literature as either halophilic or at least halotolerant bacteria, a closer analysis of the habitats of the 676 ectoine synthesizing microorganisms shows that 7% are associated with habitats exhibiting extreme conditions, 18% are associated with marine habitats, 27% are found in terrestrial habitats, 38% represent microbes associated with various types of hosts, and 8% are even found in freshwater ecosystems (Mais et al. 2020). These numbers illustrate that microorganisms with rather different physiological attributes and growth characteristics can take advantage of ectoine synthesis to cope with environmental constrains (Supplementary Figure S1).

Phylogenomic distribution of ectoine-producing microorganisms in *Archaea*

It is well-known that many halophilic *Archaea* primarily use the salt-in osmostress response. They accumulate molar concentrations of potassium chloride on a permanent basis to balance the steep osmotic gradient across their cytoplasmic membrane. This strategy necessitated, on an evolutionarily timescale, the adjustment of the amino acid composition of the entire proteome in order to keep proteins soluble and functional (Gunde-Cimerman et al. 2018). However, there are also many *Archaea* that use the salt-out response when they adjust to high salinity/osmolarity environments and they synthesize a variety of compatible solutes, some of which also serve as excellent

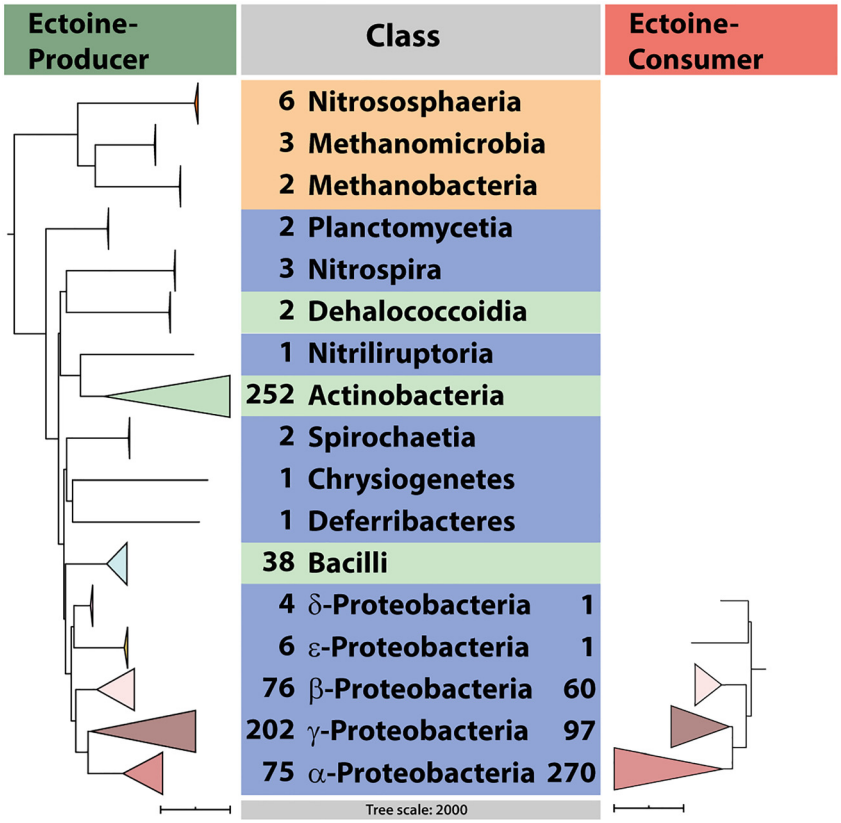

Figure 2: Phylogenetic distribution of ectoine-producing and ectoine–consuming microorganisms. The phylogenomic association of ectoine-producing and ectoine-consuming microorganisms was built on a recently reported data-set (Mais et al. 2020). In this dataset, 665 bacterial and 11 archaeal ectoine-producing microorganisms, and 429 ectoine consuming bacteria are represented. Collectively, in these 1105 microorganisms, 96 bacteria are found that both synthesize and consume ectoine. Ectoine producers were identified in the database search through the presence of the signature gene (*ectC*) for the ectoine synthase when juxtapositioned to other ectoine biosynthetic genes (*ectAB*) (Czech et al. 2018a, 2019a). Ectoine consuming bacteria were identified through the presence of juxtapositioned genes (*eutD*/*eutE*) for the central catabolic enzyme bi-module, the ectoine hydrolase (EutD) and the DABA-deacetylase (EutE) (Mais et al. 2020). For the studied 1105 microorganisms, 16S-rRNA data were collected and represented as collapsed clades. The scale is given as the number of changes between the corresponding DNA-sequences.

thermoprotectants (Müller et al. 2005; Roeßler and Müller 2001). Hence, the finding that some *Archaea* can synthesize ectoine (or 5-hydroxyectoine) is in principle per se not surprising (Widderich et al. 2016a).

Those *Archaea* that contain ectoine biosynthetic genes belong to the phyla of the Euryarchaeota and the Thaumarchaeota. Ectoine/5-hydroxyectoine production was experimentally verified in the Thamuarchaeon *Nitrosopumilus maritimus* (Widderich et al. 2016a). When one considers the ecophysiology of ectoine-producing *Archaea* identified in the previous database analysis in greater detail, one finds interesting environmental constrains that impinge on the ability to synthesize 5-hydroxyectoine, as the ectoine hydroxylase EctD is an oxygen-requiring enzyme (Bursy et al. 2007; Höppner et al. 2014; Widderich et al. 2014a). Accordingly, *ectD* is absent from the strict anaerobic Euryarchaeota, while it can be found in oxygen-tolerant nitrifying *Thaumarchaeota* (Widderich et al. 2016a). Interestingly, from the 81 *Thaumarchaeota* analyzed by Ren et al. (2019), only seven representatives possessed ectoine/5-hydroxyectoine biosynthetic genes and all of these archaea inhabit shallow ocean waters. In contrast, none of the *Thaumarchaeota* living either in deep ocean waters or in terrestrial habitats possessed ectoine biosynthetic genes (Ren et al. 2019).

Previously reported data suggest that *Archaea* containing ectoine biosynthetic genes probably have acquired them via horizontal gene transfer events (Widderich et al. 2014a, 2016a), a major driver of microbial evolution (Treangen and Rocha 2011). This conclusion was based on the finding of the signature enzyme of the ectoine biosynthetic route, the ectoine synthase EctC, in distant clades for the archaeal EctC proteins, while those derived from *Bacteria* consistently followed the phylogenetic association of the bacteria from which they were derived (Widderich et al. 2016a). We reinvestigated this issue by examining those EctC-containing *Archaea* whose genome sequences are currently available in the database of the National Center for Biotechnology Information (NCBI). Among the represented 1762 archaeal genomes, 40 presumably ectoine-producing *Archaea* were found: 10 were representatives of the *Thaumarchaeota*, 29 were *Euryarchaeota*, and a single representative of the *Crenarchaeota* was represented (Figure 3A). The phylogenomic uneven distribution of *ectC* is highlighted by the fact that among the 1762 archaeal genomes available through the NCBI database, 1155 originate from *Eury-achaeota*, yet only 29 genomes contained ectoine biosynthetic genes. This skewed distribution is also found for the *Thaumarchaeota* (10 *ectC*-containing genome sequences out of 128 database entries) and for the *Crenarchaeota* just a single *ectC*-containing genome sequence was detected out of 124 represented genomes.

We aligned the amino acid sequences of the aforementioned 40 archaeal EctC proteins with 591 bacterial EctC proteins and visualized the resulting data set through an EctC-based tree using bioinformatic resources provided by the iTOL suit (Letunic and Bork 2016). With a few exceptions, the bacterial EctC proteins followed in this type of analysis the taxonomic association of those microorganisms from which they were derived (Figure 3B). However, this was not true for the archaeal EctC proteins as these were present in three major, yet separate clusters (and five addition positions). Notably, two separate clusters of *Euryarchaeota* were present and that of the Thaumarchaeota even contained an EctC protein from the Euryarchaeon *Methanophagales* (Figure 3B). Although this type of analysis certainly does not formally prove the involvement of gene transfer events, horizontal gene transfer seems a plausible scenario for the evolution of those *Archaea* containing *ect* gene clusters. It is well known that transfer of bacterial genes into *Archaea* significantly contributed to the development and expansion of this major domain of life (Wang et al. 2019).

Biochemistry and structural biology of ectoine biosynthesis

The overall route and enzymes for the synthesis of ectoine and 5-hydroxyectoine are known for some time (Bursy et al. 2007; Ono et al. 1999; Peters et al. 1990; Reshetnikov et al. 2011b) (Figure 4). Biosynthesis of ectoines is an energy-demanding process. Calculations by A. Oren (1999) suggest that the production of just a single ectoine molecule requires the expenditure of about 40 ATP equivalents. This calculation considers the entire production route of ectoine in cells growing heterotrophically with glucose at the sole carbon and energy source. The metabolism of glucose would otherwise yield 38 ATP-equivalents and two additional ATP-equivalents have to be spent to produce a single ectoine molecule from the biosynthetic precursor L-asparate-β-semialdehyde via the EctABC enzymes (Oren 1999). Hence, in these calculations, the vast majority of the spent ATP equivalents are required for the synthesis of the ectoine precursor L-asparate-β-semialdehyde. Energetic calculations based on a theoretical metabolic network analysis of *Halomonas elongata* suggest that either (i) an energetically neutral or (ii) an even one ATP-generating route can also be considered (Schwibbert et al. 2011). By growing *H. elongata* continuously in a calorimeter with

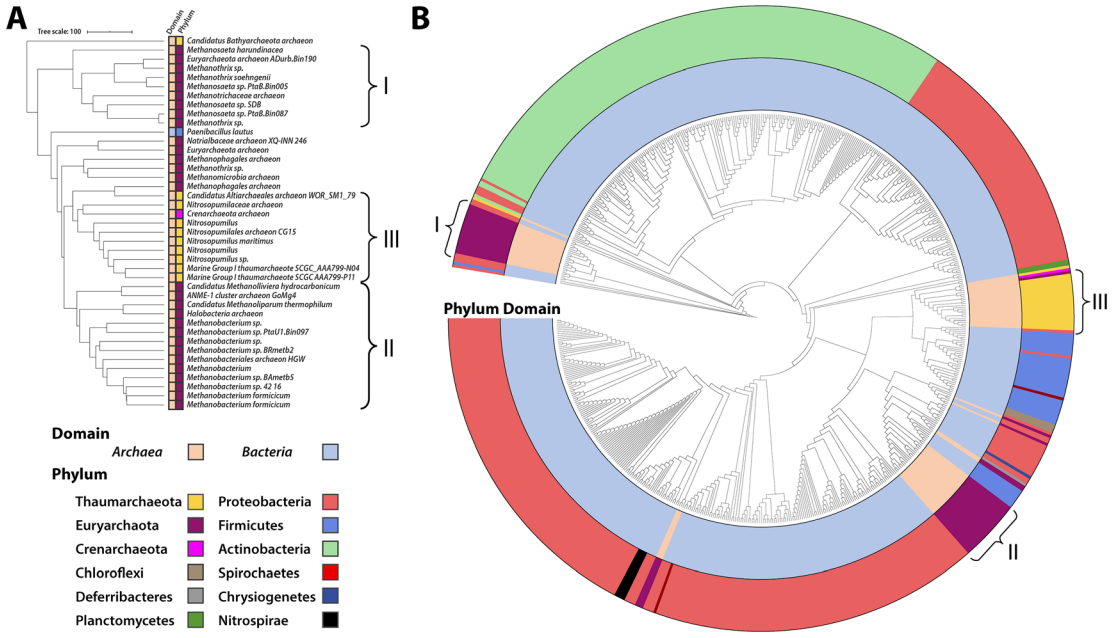

Figure 3: Phylogenetic analysis of EctC-type proteins. (A) 40 EctC-type proteins from *Archaea* were identified in the NCBI database using the EctC ectoine synthase from *Paenibacillus* lautus as the search query. The amino acid sequences of these proteins were aligned using Clustal O (Sievers et al. 2011) and the data from this alignment are graphically represented using the iTOL tool (Letunic and Bork 2016). Phylogenetic affiliations of microorganisms to different phyla are shown by color-code as listed in the figure. The scale of the tree is given as amino acid changes between the sequences. (B) The 40 archaeal EctC-type proteins found in the NCBI database were added to the previously assembled dataset of EctC-type proteins by Czech et al. (2018). The protein sequences were aligned with Clustal O (Sievers et al. 2011) and graphically represented with the iTOL tool (Letunic and Bork 2016). Phylogenetic affiliations of microorganisms to different domains and phyla are shown by color-code as listed at the bottom of the Figure. For simplicity, the names of the *ectC*-possessing microorganisms were left out.

glucose as the carbon and energy source, an approximately 100% efficiency for the conversion of the substrate glucose into ectoine was achievable (Maskow and Babel 2001).

Great strides have recently been made in our understanding of this biosynthetic process through a detailed biochemical and structural analysis of all enzymes involved in ectoine/5-hydroxyectoine production. In the following sections, we summarize our structural and biochemical understanding of the enzymes forming the biosynthetic pathway for ectoine and its derivative 5-hydroxyectoine (Figure 4, left side).

Making the precursor for ectoine biosynthesis

L-aspartate-β-semialdehyde is a central metabolic hub that feeds a branched network for the biosynthesis of several amino acids, the production of crucial components of the peptidoglycan and of the spore coat, and the synthesis of antibiotics (Lo et al. 2009). L-aspartate-β-semialdehyde is also the precursor for the synthesis of ectoine (Ono et al. 1999; Peters et al. 1990) (Figure 4). Two enzymes are involved in L-aspartate-β-semialdehyde production from L-glutamate, the aspartate kinase (Ask) (EC 2.7.2.4) and the aspartate-semialdehyde-dehydrogenase (Asd) (EC 1.2.1.11). Ask catalyzes the ATP-dependent phosphorylation of L-Aspartate to yield ADP and L-aspartyl-β-phosphate, the latter of which then undergoes a reductive dephosphorylation in a NADP-dependent reaction to form L-aspartate-β-semialdehyde (Figure 4).

We know of only one ectoine/5-hydroxyectoine biosynthetic gene cluster in which genes for Ask and Asd enzymes are jointly present (Czech et al. 2018a; Widderich et al. 2014a). This gene cluster (*ask_ect-asd-ectABCD*) is found in *Kytococcus sedentarius*, a marine, strictly aerobic Gram-positive bacterium (Sims et al. 2009). Using molecular modeling, we have visualized the overall fold of the *K. sedentarius* Ask and Asd enzymes using the crystal structure of the Ask protein from *Synechocystis* sp. PCC 6803 (PDB accession code: 3L76) and that of the Asd protein from *Mycobacterium tuberculosis* (PDB accession code: 3TZ6) as templates (Robin et al. 2010; Vyas et al. 2012). The Ask_Ect and Asd proteins from *K. sedentarius* are both predicted to form homo-dimers (Figure 5).

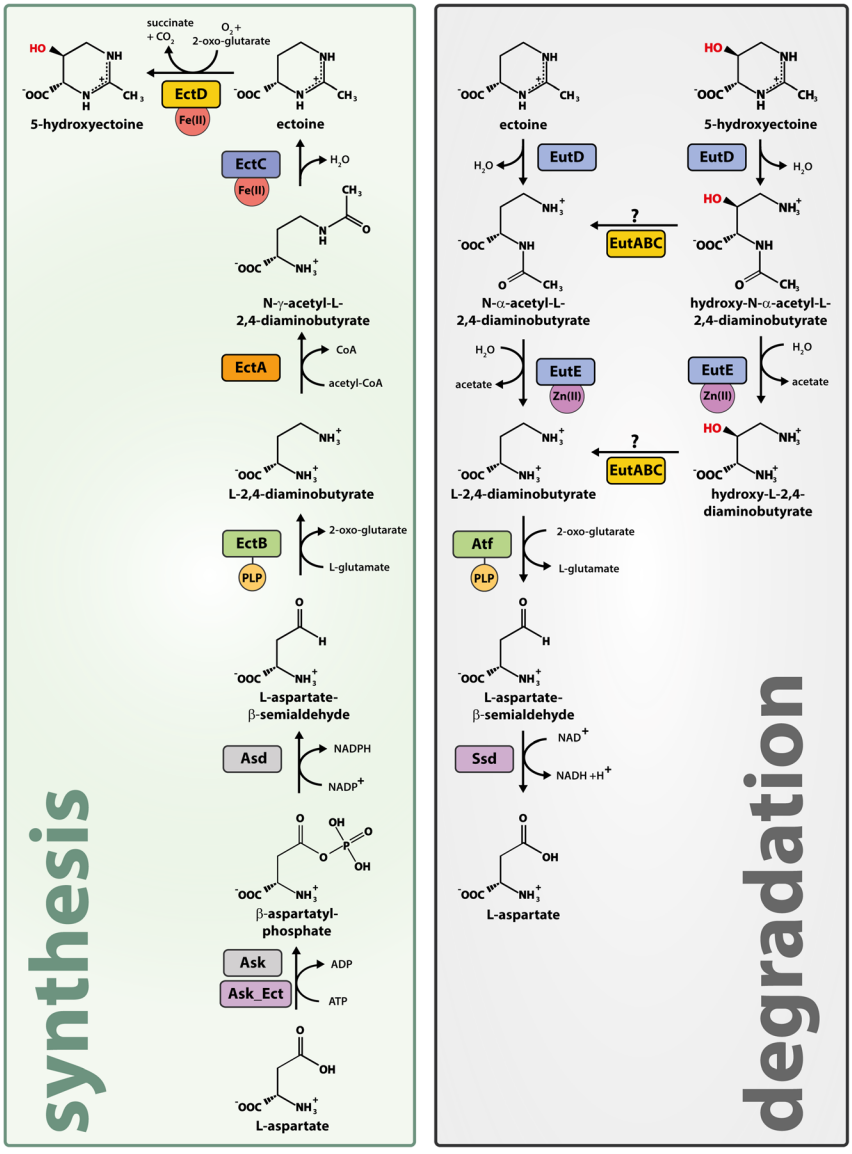

Figure 4: Ectoine metabolism. The green and grey boxes show the pathways enabling biosynthesis and degradation of ectoine/5-hydroxyectoine, respectively. Both can be thought as opposites of one another. Note: The biosynthesis pathway employs γ-ADABA as unique intermediate, while the degradation pathway relies on the α-ADABA. As discussed in the text, the latter isomer is critical for the genetic regulation of ectoine metabolism.

While a gene for Asd is typically absent from *ect* biosynthetic gene clusters, many contain a gene for a specialized aspartokinase (Ask_Ect) (Czech et al. 2018a; Lo et al. 2009; Reshetnikov et al. 2006; Widderich et al. 2014a). Within the super-family of aspartokinases, Ask_Ect-type enzymes form a separate branch and its representatives are mainly found in the Alpha-, Gamma- and Delta-Proteobacteria (Lo et al. 2009). Aspartokinases are enzymes with a complex allosteric activity control (Lo et al. 2009), and this is also true for Ask_Ect (Stöveken et al. 2011). A considerable number of microorganisms contain genes for multiple aspartokinases, and as exemplified by Ask_Ect, some of which then serve specialized biosynthetic routes (Lo et al. 2009). Often, multiple *ask* genes present in a given bacterium are also subject to different types of genetic regulation thereby tying enhanced synthesis of specialized Ask enzymes to the needs of the particular biosynthetic route they assist (Lo et al. 2009).

In an assessment of the gene content of 582 *ect* biosynthetic gene clusters, 23% (133) possessed an associated *ask_ect* gene (Czech et al. 2018a). It thus appears that while a specialized aspartokinase seems to be beneficial for ectoine/5-hydroxyectoine production (Bestvater et al. 2008; Stöveken et al. 2011), its presence is certainly not a pre-requisite to achieve adequate osmostress-protective levels of ectoines through synthesis. However, as *ask_ect* genes are co-expressed along with the ectoine/5-hydroxyectoine biosynthetic genes, osmotically stressed cells will ramp-up the production of this specialized aspartokinases enabling them to seemingly avoid a

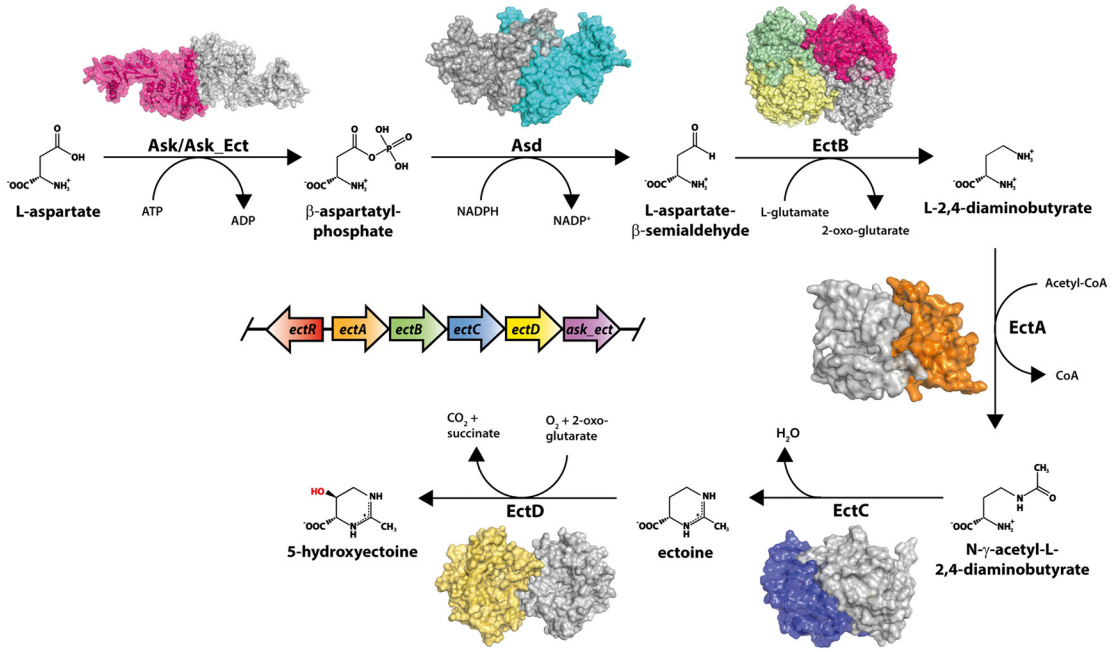

Figure 5: Structural enzymology of ectoine/5-hydroxyectoine biosynthesis. L-aspartate is transformed to ß-aspartylphosphate by either a general aspartokinase (Ask), or a specialized aspartokinases (Ask_Ect) (Stöveken et al. 2011). The shown structure of the Ask_Ect from *K. sedentarius*, a marine actinobacterium and opportunistic pathogen (Sims et al. 2009), was modeled on a crystallized aspartate kinase from *Synechocystis* sp. PCC 6803 (PDB: 3L76) (Robin et al. 2010). The intermediate ß-aspartylphosphate is then further transformed to L-aspartate-ß-semialdehyde by an aspartate semialdehyde dehydrogenase (Asd). The Asd protein originating from the *ect* cluster of *K. sedentarius* was modeled on the aspartate semialdehyde dehydrogenase from *M. tuberculosis* (PDB: 3TZ6) (Vyas et al. 2012). The ectoine/5-hydroxyectoine biosynthetic gene cluster (*ask_ect-asd-ectABCD*) from *K. sedentarius* is the only one of which we are aware in which the genes for the two enzymes required for the synthesis of the ectoine biosynthetic precursor, L-aspartate-ß-semialdehyde, are jointly present. L-2,4-diaminobutyric acid (DABA) aminotransferase EctB: (PDB: 6RL5); DABA-acetyltransferase EctA (PDB: 6SLL); ectoine synthase EctC (PDB: 5ONM), ectoine hydroxylase EctD (PDB: 4Q5O). Details on the features of these crystal structures are given in Table 1. In the middle of the figure, the genetic organization of the *ect* gene cluster from *Acidiphilium cryptum* is shown (Moritz et al. 2015). The *ectR* gene (shown in red) encodes a MarR-type repressor protein (EctR) that is involved in the transcriptional control of a considerable number of ectoine biosynthetic gene clusters (Czech et al. 2018a; Mustakhimov et al. 2010).

metabolic bottleneck for the supply of the ectoine biosynthetic precursor L-aspartate-β-semialdehyde (Figure 5). Indeed, the productivity of a heterologous *Escherichia coli* cell factory carrying the *Pseudomonas stutzeri* A1501 *ectABCD-ask_ect* operon cluster was notably higher when the *ask_ect* gene was present on the recombinant plasmid (Stöveken et al. 2011). Such a beneficial effect on ectoine production had also been seen when the *Marinococcus halophilus ectABC* genes were co-expressed in *E. coli* along with the gene for a feed-back resistant Ask_LysC enzyme derived from *Corynebacterium glutamicum* (Bestvater et al. 2008). A feed-back resistant Ask_LysC is also part of the genomic chassis of the current ectoine-producing champion (65 g L^{-1}), a synthetic cell factory of the industrial workhorse *C. glutamicum* (Becker et al. 2013; Giesselmann et al. 2019).

The Ask_Ect enzyme from the plant roots associated bacterium *P. stutzeri* A1501 is so far the only representative of this specialized group of aspartokinases that has been studied biochemically at any level of detail (Stöveken et al. 2011). The Ask_Ect enzyme and that of the only other aspartokinase (Ask_LysC) present in from *P. stutzeri* A1501 possess an amino acid sequence identity of 25% and display a different domain organization, thereby reflecting membership of the two Ask proteins in different subgroups of the Ask enzyme superfamily (Lo et al. 2009). When biochemically benchmarked against each other, comparable kinetic parameters for the two enzymes were found, both with respect to the K_m and V_{max} values for their substrates L-aspartate and ATP (e.g., the K_m for L-aspartate were about 22 mM for Ask_Lys and 30 mM for the Ask_Ect enzyme; the corresponding V_{max} values were about 5 U mg^{-1} and about 7 U mg^{-1}, respectively) (Stöveken et al. 2011). These enzyme activities are comparable to those of other biochemically characterized microbial aspartokinases (Lo et al. 2009).

Most notably were the differences in the allosteric control of Ask_LysC and Ask_Ect. Ask_LysC was feed-back inhibited by L-threonine and in a concerted fashion by L-threonine and L-lysine, while Ask_Ect was only inhibited by L-threonine. The two enzymes additionally differed in their allosteric control when they were assayed in the presence of high concentrations of salts (KCl, NaCl). High salt concentrations reduced the allosteric inhibition Ask_Ect by L-threonine, as the IC_{50} value was increased from 3.6 to 18.7 mM and 13.5 mM in the presence of 650 mM NaCl or KCl, respectively (Stöveken et al. 2011). This behavior of the Ask_Ect enzyme might be relevant under temporary suddenly increased ionic strength conditions in the cytoplasm of osmotically stressed ectoine-producing cells. Remarkably, the feed-back inhibition of Ask-LysC enzyme activity by either L-threonine alone, or a combination of L-threonine and L-lysine, was not influenced by high concentrations of either KCl or NaCl (Stöveken et al. 2011).

The L-2,4-diaminobutyrate transaminase: EctB

The first step of ectoine biosynthesis is catalyzed by the L-2,4-diaminobutyrate transaminase, EctB (EC 2.6.1.76) (Figure 4). EctB uses L-glutamate as the amino donor and L-aspartate-β-semialdehyde as the acceptor molecule to form 2-oxoglutarate and diamino-butyric acid (DABA), the latter serving as substrate for EctA (Hillier et al. 2020; Ono et al. 1999; Reshetnikov et al. 2011b; Richter et al. 2019) (Figure 4). EctB enzymes belong to the pyridoxal-5′-phosphate (PLP)-dependent transaminases (also referred to in the literature as aminotransferases) (Steffen-Munsberg et al. 2015), with the *γ*-aminobutyrate transaminase (GABA-TA) being the closest homologue (Bruce et al. 2012; Richter et al. 2019). Indeed, EctB from the thermotolerant Gram-positive bacterium *Paenibacillus lautus* possesses a residual GABA-TA activity (Richter et al. 2019). Structural, computational and biochemical analysis of EctB proteins from the Gram-negative bacterium *Chromohalobacter salexigens* and from *P. lautus*, show that the protein forms tetramers (Hillier et al. 2020; Richter et al. 2019) (Figure 5). Earlier biochemical studies on the *H. elongata* and *Methylomicrobium alcaliphilum* EctB enzymes suggested that these proteins form hexamers (Ono et al. 1999; Reshetnikov et al. 2006, 2011b). In the structure of the *C. salexigens* EctB tetramer (Table 1) (Figure 6A), each of the monomers carries a PLP molecule covalently attached to a Lys residue (Hillier et al. 2020) (Supplementary Figure S2). PLP is critical for enzyme function as substitution of this Lys residue with amino acid residues unable to covalently bind PLP yielded enzymatically inactive variants of the *P. lautus* EctB protein (Richter et al. 2019).

Structural analysis of *C. salexigens* EctB also revealed the molecular determinants required for the interactions of the monomers in the tetrameric assembly, including the positioning of a gating loop that aids the proper formation of the active site of the neighboring monomer (Hillier et al. 2020) (Supplementary Figure S2). Transaminases are known to possess two binding pockets for the amino donor and the acceptor molecule that are required for catalytic

Table 1: Structures of ectoine and 5-hydroxyectoine biosynthetic enzymes.

Enzyme (EC number)	Ligand	PDB accession	Resolution	Organism	References
EctA	apo	6SLK	2.2 Å	*P. lautus*	(Richter et al. 2020)
(2.3.1.178)	CoA	6SK1	1.5 Å	*P. lautus*	(Richter et al. 2020)
	DABA – CoA	6SLL	1.2 Å	*P. lautus*	(Richter et al. 2020)
	DABA	6SL8	1.5 Å	*P. lautus*	(Richter et al. 2020)
	N-γ-ADABA	6SJY	2.2 Å	*P. lautus*	(Richter et al. 2020)
EctB (2.6.1.76)	PLP	6RL5	2.5 Å	*C. salexigens*	(Hillier et al. 2020)
EctC (4.2.1.108)	apo; semi-closed	5BXX	2.0 Å	*S. alaskensis*	(Widderich et al. 2016b)
	1,2-Propanediol; open	5BY5	1.2 Å	*S. alaskensis*	(Widderich et al. 2016b)
	Fe(III)	5ONM	1.52 Å	*P. lautus*	(Czech et al. 2019a)
	Fe(III); *N*-γ-ADABA	5ONN	1.4 Å	*P. lautus*	(Czech et al. 2019a)
	Fe(III); ectoine	5ONO	2.5 Å	*P. lautus*	(Czech et al. 2019a)
EctD	Fe(III)	3EMR	1.9 Å	*V. salexigens*	(Reuter et al. 2010)
(1.14.11.55)	apo	4MHR	2.1 Å	*S. alaskensis*	(Höppner et al. 2014)
	Fe(III)	4MHU	2.6 Å	*S. alaskensis*	(Höppner et al. 2014)
	Fe(III); 2-oxoglutarat; 5-hydroxyectoine	4Q5O	1.6 Å	*S. alaskensis*	(Höppner et al. 2014)

The abbreviations used are: CoA: Coenzyme A; DABA: diaminobutyric acid; *N*-γ-ADABA: *N*-γ-acetyl-diaminobutyric acid; PLP: pyridoxal-5-phosphate.

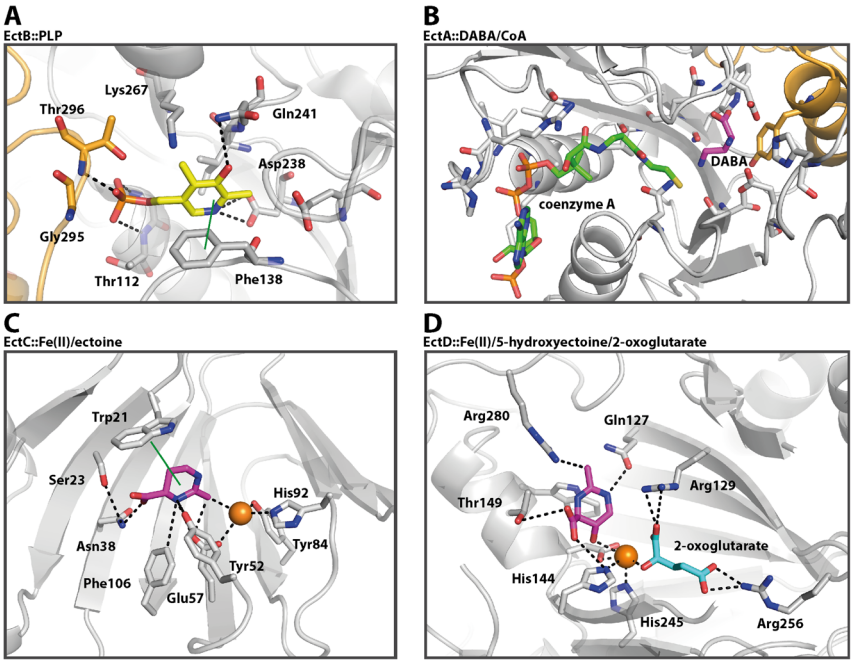

Figure 6: Structural views into the active sites of ectoine/5-hydroxyectoine biosynthetic enzymes. (A) The crystal structure of the dimeric EctB enzyme from *C. salexigens* was solved in complex with the cofactor PLP (yellow sticks) which is tightly coordinated by amino acid residues originating from both monomers, highlighted as orange (monomer 1) and grey sticks (monomer 2) (PDB: 6RL5) (Hillier et al. 2020). (B) The EctA enzyme from *P. lautus* was crystallized in complex with DAB (PDB: 6SL8) and CoA (PDB: 6SK1). An overlay of both structures with CoA depicted as green and DAB as pink sticks is shown. The DAB binding site also involves a Tyr-residue from the second monomer (shown as an orange stick) (Richter et al. 2020). (C) The ectoine synthase EctC from *P. lautus* is shown in complex with ectoine (pink sticks); the amino acid residues involved in ectoine and iron binding are shown as sticks and iron is represented by an orange sphere (PDB: 5ONO) (Czech et al. 2019a). (D) The ectoine hydroxylase, EctD, from *S. alaskensis* is depicted in complex with the reaction production 5-hydroxyectoine (pink sticks) and the co-substrate 2-oxoglutarate (blue sticks). Amino acids involved in ligand binding are shown as grey sticks, and the iron catalyst is represented by an orange sphere (PDB: 4Q5O) (Höppner et al. 2014).

activity (Steffen-Munsberg et al. 2015). The crystal structure of the *C. salexigens* EctB protein provides the first detailed description of the architecture of these binding sites of L-2,4-diaminobutyrate transaminase that are involved in ectoine production (Hillier et al. 2020). However, so far crystal structures in complex with the substrates (L-glutamate as the amino donor and L-aspartate-β-semialdehyde as the amino acceptor), or the reaction products (2-oxoglutarate and DABA) are not available. Such structures are highly desirable as they should further strengthen our understanding of the catalytic activity of EctB.

The diaminobutyrate acetyltransferase: EctA

The second step in ectoine biosynthesis is catalyzed by L-2,4-diaminobutuyrate acetyltransferase (EC 2.3.1.178), EctA (Figure 4). This enzyme catalyzes the acetylation of L-2,4-diaminobutyric acid (DABA) into *N*-γ-acetyl-L-2,4-diaminobutyric acid (*N*-γ-ADABA) in an acetyl-CoA-dependent manner. EctA belongs to the GCN5-related *N*-acetyltransferases (GNAT) (Salah Ud-Din et al. 2016; Vetting et al. 2005). High-resolution X-ray structures of the dimeric EctA from *P. lautus* in its apo-, substrate, and co-substrate-bound forms have recently been reported, and a crystal structure trapping the reaction product of EctA, *N*-γ-ADABA, has also been obtained (Richter et al. 2020) (Table 1). These crystal structures, combined with biochemical analysis, clarified the entire reaction mechanism catalyzed by the EctA enzyme. The enzyme activity of the homodimeric *P. lautus* EctA protein (Figure 5) is highly region-specific as it produces exclusively *N*-γ-ADABA but not the isomer *N*-α-ADABA (Richter et al. 2020), a crucial metabolite formed during ectoine catabolism (see below).

In the apo-form of the enzyme, the binding sites for the substrate DABA and the co-substrate acetyl-CoA are present in an "open" conformation. In this structure, there is a surface-exposed extended tunnel in which acetyl-CoA will bind, and a deep cavity in which DABA will be bound (Supplementary Figure S3). In the secondary complexes of the *P. lautus* EctA protein [*Pl*EctA::CoA and *Pl*EctB::DABA] (Figure 6B), the chemical groups of the substrate and of the co-substrate involved in the acetylation reaction point towards each other. A structure with *N*-γ-ADABA was also captured (Richter et al. 2020) (Supplementary Figure S3). While keeping in mind that crystal structures only provide snapshots of "trapped states" of proteins, the superimposable positions of DABA and *N*-γ-ADABA in the active site suggest that the backbone of the EctA protein and the amino acid side chains relevant for substrate, co-substrate and product binding do not move substantially during enzyme catalysis. An overlay of the *Pl*EctA::DABA and *Pl*EctA::CoA structures revealed a distance of less than 3 Å between the sulfur atom of the CoA molecule and the

reactive nitrogen in the γ-position of DABA. Hence, these two crystal structures probably represent stages of the L-2,4-diaminobutuyrate acetyltransferase prior to catalysis (Richter et al. 2020). Because acetyl-CoA is highly reactive, the non-reactive CoA was purposely used in the crystallization of EctA. However, when the thiol hydrogen of CoA was substituted *in silico* with an acetyl group, it became clear how close the reactive groups of acetyl-CoA and DABA are juxtapositioned just before the transfer of the acetyl group to DABA occurs (Supplementary Figure S3). Collectively, the obtained five crystal structures of the *P. lautus* EctA protein (Table 1) allowed the rendering of a movie capturing the various enzymatic steps of the L-2,4-diaminobutuyrate acetyltransferase conducted between the binding of the substrate and co-substrate and the formation of the reaction product (Richter et al. 2020).

The ectoine synthase: EctC

The ectoine synthase EctC (EC 4.2.1.108) uses the EctA-produced *N*-γ-ADABA to synthesize ectoine in an iron-dependent cyclo-condensation reaction in which a water molecule is eliminated (Czech et al. 2019a; Widderich et al. 2016b) (Figure 4). Specifically, EctC cyclizes the linear *N*-γ-ADABA molecule through a nucleophilic attack on the alpha amino nitrogen positioned at the terminal carbonyl group of the substrate (Czech et al. 2019a; Ono et al. 1999; Witt et al. 2011). High-resolution crystal structures of EctC proteins from the cold-adapted bacterium *Sphingopyxis alaskensis* and the thermotolerant bacterium *P. lautus*, have been reported in the absence or presence of various ligands (Czech et al. 2019a; Widderich et al. 2016b) (Table 1). The crystal structures of the EctC protein from both microorganisms show that the ectoine synthase belongs to the cupin super-family (Figure 5). These type of proteins are characterized by barrel-like structures in which the catalytically important residues protrude into the lumen of the barrel (Dunwell et al. 2004) (Supplementary Figure S4). Most of these proteins contain catalytically important divalent transition state metals (e.g., iron, copper, zinc, manganese, cobalt, nickel), allowing the imposition of different types of chemical reactions onto a common structural fold (Dunwell et al. 2004). The EctC protein forms homodimers with a 'head-to-tail' configuration through backbone-contacts and weak hydrophobic interactions mediated by two beta-sheets within each monomer (Widderich et al. 2016b; Czech et al. 2019a). Crystal structures of *P. lautus* EctC in its iron-bound, iron- and substrate (*N*-γ-ADABA)-bound and in the presence of its reaction product ectoine (Figure 6C) have been determined (Table 1) (Supplementary Figure S4), which enabled reconstruction of its catalytic cycle (Czech et al. 2019a). The *N*-γ-ADABA substrate appears in these structures an in extended configuration, rather than in a pre-bent form that would facilitate the EctC-mediated cyclo-condensation reaction (Czech et al. 2019a) (Figure 5). Residues critical for iron coordination, *N*-γ-ADABA- and ectoine-binding are evolutionarily highly conserved, and their mutation leads to EctC variants with only residual catalytic activity (Czech et al. 2019a; Widderich et al. 2016b).

In a slow side-reaction, EctC can also produce the synthetic compatible solute 5-amino-3,4-dihydro-2H-pyrrole-2-carboxylate (ADPC) by cyclic condensation of glutamine (Witt et al. 2011). Import of ADPC by an osmotically sensitive *H. elongata* mutant affords osmostress resistance. ADPC also possesses effective chemical chaperone activity for a model enzyme subjected to denaturing repeated freeze-thaw cycles (Witt et al. 2011). Of note is also that the substrate for the ectoine synthase, *N*-γ-ADABA, is an osmostress protectant and function-preserving compatible solute in its own right (Canovas et al. 1999).

EctC can be regarded as the signature enzyme for ectoine biosynthesis, because EctB-related transaminases and EctA-related acetyl-transferases have counterparts in other metabolic pathways of microbes. Database searches using EctC as the query protein can thus be used to identify *bona fide* ectoine biosynthetic gene clusters with confidence (Czech et al. 2018a; Leon et al. 2018; Widderich et al. 2014a). However, EctC-type proteins can also be found in microorganisms either lacking the *ectAB* genes or containing *ectC*-type gene copies in addition to complete *ectABC(D)* operons. Orphan *ectC*-type genes were first identified in the plant pathogen *Pseudomonas syringae* pv. *syringae* (Kurz et al. 2010). In an EctC-based phylogenetic tree, the orphan EctC-type proteins are found in a separate group located at the root of the tree representing a taxonomically rather heterogeneous assemblage of microorganisms (Czech et al. 2018a). *bona fide* EctC proteins and their orphan counterparts share considerable amino acid sequence identity (around 40%) with essentially all amino acid residues critical for substrate and metal binding being conserved. However, the physiological role of orphan EctC-type proteins awaits biochemical clarification.

The ectoine hydroxylase: EctD

EctD (EC 1.14.11.55) catalyzes the iron-dependent hydroxylation of ectoine into its 5-hydroxylated counterpart using molecular oxygen and 2-oxoglutarate as the substrates. In addition to 5-hydroxyectoine, EctD also forms CO_2 and

succinate as products during catalysis (Figure 4). EctD proteins form 'head-to-tail' arranged homo-dimers in solution (Figure 5) and in various crystal structures (Table 1) (Höppner et al. 2014; Reuter et al. 2010; Widderich et al. 2014a). Crystal structures of the ectoine hydroxylase from the salt-tolerant bacterium *Virgibacillus salexigens* and the cold-adapted marine bacterium *S. alaskensis* in its apo-form, and various ligands have been reported (Höppner et al. 2014; Reuter et al. 2010). One of these structures jointly contained the iron catalyst, the co-substrate 2-oxoglutarate, and the reaction product 5-hydroxyectoine (Figure 6D). Collectively, the crystallographic analysis, coupled with site-directed mutagenesis and molecular dynamics simulations, exposed an intricate network of interactions between the enzyme and its ligands that ensures the placing of ectoine within the active site such that its hydroxylation can occur in a precise position- and stereo-specific manner (Höppner et al. 2014).

The overall fold of EctD consists of a double-stranded β-sheet core surrounded and stabilized by a number of α-helices (Supplementary Figure S5). This core, also known as the jelly role or cupin fold (Aik et al. 2012; Hangasky et al. 2013; Islam et al. 2018), is formed by two four-stranded anti-parallel β-sheets that are arranged in the form of a *β*-sandwich. The two monomers in the EctD dimer assembly interact via amino acid residues located in extended loop areas (Höppner et al. 2014). These structural characteristics also assign EctD to the cupin super-family (Höppner et al. 2014; Reuter et al. 2010). Ectoine hydroxylases contain an evolutionarily highly conserved signature sequence consisting of a continuous stretch of 17 amino acids (F-X-W-H-S-D-F-E-T-W-H-X-E-D-G-M/L-P). This segment is structurally and functionally important as it spans an extended α-helix and a linked short β-sheet lining one side of the cupin barrel. It harbors five residues involved in the binding of the iron catalysts, the co-substrate 2-oxoglutarate, and the reaction product 5-hydroxyectoine (Höppner et al. 2014; Reuter et al. 2010; Widderich et al. 2014a). Furthermore, it can be used as a signature sequence to distinguish *bona fide* ectoine hydroxylases from other members of the widely distributed non-heme-containing iron (II) and 2-oxoglutarate-dependent family dioxygenases, as *bona fide* ectoine hydroxylases are often miss-annotated in microbial genome sequences as phytanoly-CoA dioxygenases.

EctD belongs to the sub-family of non-heme-containing iron (II) and 2-oxoglutarate-dependent dioxygenases (Aik et al. 2012; Dunwell et al. 2004; Hangasky et al. 2013; Islam et al. 2018). These types of enzymes carry out a remarkably broad range of oxidative reactions on a variety of substrates, yet common enzyme reaction mechanisms are observed. Most of these types of dioxygenases couple a two-electron oxidation of their substrates with the chemical reactivity of oxygen and 2-oxoglutarate (Hangasky et al. 2013; Herr and Hausinger 2018; Islam et al. 2018). The ectoine hydroxylase adheres to this general reaction scheme (Widderich et al. 2014b) and seems to closely follow in its catalytic cycle that of the biochemically and structurally intensively studied archetypical non-heme-containing iron (II) and 2-oxoglutarate-dependent dioxygenase, the taurine dioxygenase TauD (Proshlyakov et al. 2017).

Within the cupin domain of EctD, the iron atom coordinates through two conserved histidines and an aspartate (Höppner et al. 2014; Reuter et al. 2010) (Figure 6D). These residues belong to the structurally conserved *H-X-(D/E)…H* motif, the so-called '2-His-1-carboxylate facial triad', forming a type of mononuclear iron center found in many members of the dioxygenase superfamily (Aik et al. 2012; Hangasky et al. 2013). The 2-oxoidic group of the co-substrate 2-oxoglutarate locates in close vicinity to the iron catalysts (Höppner et al. 2014) (Supplementary Figure S6). The 5-hydroxyectoine molecule found in the *S. alaskensis* EctD structure (PDB: 4Q5O) (Table 1) is bound slightly above the three residues forming the iron binding site with its hydroxyl group at C-5 in the heteropyrimidine ring pointing towards the iron catalysts. It is also positioned in close vicinity of the co-substrate 2-oxoglutarate, thereby resembling its expected orientation in the active site cavity after the enzymatic hydroxylation of ectoine by EctD (Höppner et al. 2014).

The EctD-mediated hydroxylation of ectoine occurs in a precise position and stereospecific manner yielding exclusively, both *in vivo* and *in vitro*, (4*S*,5*S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid (=5-hydroxyectoine) (Bursy et al. 2007; Czech et al. 2016; Inbar and Lapidot 1988; Inbar et al. 1993). So far, nine ectoine hydroxylases have been characterized biochemically, including enzymes from various extremophilic *Bacteria* and also one protein from a marine archaeon (*Nitrosopumilus maritimus*) (Bursy et al. 2007; Reuter et al. 2010; Widderich et al. 2014a, 2016a). These enzymes seemed to possess similar kinetic parameters, but the subsequent optimization of assay conditions for individual enzymes revealed significant differences in their catalytic efficiency (Czech et al. 2019b). This aspect becomes important when the catalytic efficiency and robustness of ectoine hydroxylases are benchmarked against each other when EctD enzymes are used in chemical biology approaches to hydroxylate substrates other than ectoine (Czech et al. 2016, 2019b; Galinski et al. 2009; Hara et al. 2019).

As a mark of their evolutionary history, many enzymes exhibit a certain degree of substrate ambiguity. This

metabolic profile, also referred to as underground metabolism, can be exploited by microbial cells to develop novel functions (D'Ari and Casadesus 1998; Jensen 1976). Recent studies have already exploited the substrate promiscuity of the EctD enzyme towards ectoine-related substrates; e. g., aiming at the selective hydroxylation of L-proline and of the synthetic ectoine derivative homoectoine (Czech et al. 2019b; Galinski et al. 2009; Hara et al. 2019). Hence, ectoine hydroxylases have already found interesting uses in chemical biology.

Catabolism of ectoine and 5-hydroxyectoine

Ecophysiological role of ectoines as nutrients

Synthesis of compatible solutes such as the ectoines requires considerable biosynthetic and energetic resources (Oren 1999) (see above). It is thus not surprising that microorganisms have found ways to re-use these compounds when they are no longer needed as osmostress protectants (Welsh 2000). Compatible solutes are released into the environment through the transient opening of mechanosensitive channels when producer cells are subjected to a rapid osmotic down-shift (Booth 2014). Microorganisms also excrete compatible solutes under steady-state high osmolarity growth conditions, perhaps in an effort to fine-tune turgor (Czech et al. 2016, 2018b; Grammann et al. 2002; Hoffmann et al. 2012; Lamark et al. 1992; Vandrich et al. 2020). An ecophysiologically important process for the release of compatible solutes into the environment is also cell lysis; e.g., after the attack of ectoine producers by lytic phages (Van Goethem et al. 2019). Combined, these processes can shape the ecophysiology of microbial communities and the high turn-over of compatible solutes in the soil indicates their rapid re-use in natural settings (Warren 2019).

Use of ectoines as nutrients has been described for various microorganisms (Reshetnikov et al. 2020; Schulz et al. 2017b; Schwibbert et al. 2011; Vargas et al. 2006). Indeed, free ectoines have been found in various ecosystems; e.g., in the soil and in the effluent of an acid mine (Bouskill et al. 2016; Mosier et al. 2013). Since compatible solutes are present in the environment in very low concentrations, high affinity transporters are needed to scavenge them for their use as nutrients. So far, two substrate-inducible uptake systems for environmental ectoines have been studied that supply these nitrogen-rich molecules (Figure 1) to the cell for catabolism. These are EhuABCD and UehABC, representatives of binding-protein-dependent ABC-transporters (Ehu) and tripartite ATP-independent periplasmic transporters (TRAP-T) (Ueh), respectively (Hanekop et al. 2007; Jebbar et al. 2005; Lecher et al. 2009) (Supplementary Figure S6). EhuB and UehA, the periplasmic high-affinity substrate-binding proteins have been crystalized in the presence of ectoines, thereby revealing the molecular determinants for the efficient capturing of environmental ectoines by transporters (Hanekop et al. 2007; Lecher et al. 2009) (Supplementary Figure S6). The osmotically inducible TeaABC transporter form *H. elongata*, a system closely related to the TRAP-T UehABC system from *Ruegeria pomeroyi*, not only serves for the import of ectoines as osmostress protectants and as a recycling systems for newly synthesized and excreted ectoines (Grammann et al. 2002; Kuhlmann et al. 2008b; Vandrich et al. 2020) but it might also be used for their acquisition as nutrients (Schwibbert et al. 2011).

Phylogenomics of ectoine and hydroxyectoine catabolic genes

Building on the inspection of 8557 bacterial and 293 archaeal completely sequenced genomes, Mais et al. (2020) recently identified 429 potential ectoine/5-hydroxyectoine consumers (Mais et al. 2020) (Figure 2). These were recognized through the presence of adjacent genes (*eutD*/*eutE*) for the ectoine hydrolase (EutD) (EC 3.5.4.44) and the *N*-acetyl-2,4-diaminobutyric acid (α-ADABA) deacetylase (EutE) (EC 3.5.1.125). These two proteins form the central enzyme bi-module for the catabolism of ectoines (Mais et al. 2020; Reshetnikov et al. 2020; Schulz et al. 2017b; Schwibbert et al. 2011) (Figure 4). None of the potential ectoine/5-hydroxyectoine consumers belong to the domain of the *Archaea*, and those found in the *Bacteria* are all members of the super-phylum of the Proteobacteria (Figure 2). The data on the predicted ectoine/5-hydroxyectoine consumers compiled by Mais et al. (2020) are constrained by the used database search criterion [adjacently located *eutD-eutE* genes] and thereby miss the detection of those ectoine/5-hydroxyectoine catabolic gene clusters in which these two genes are not juxtapositioned; e.g., those from *M. alcaliphilum* (Figure 7A) and related species (Reshetnikov et al. 2020). Given that ectoine/5-hydroxyectoine catabolic gene clusters are quite variable in gene order and content (Figure 7A), microorganisms that consume ectoines might occur more frequently than suggested (Mais et al. 2020).

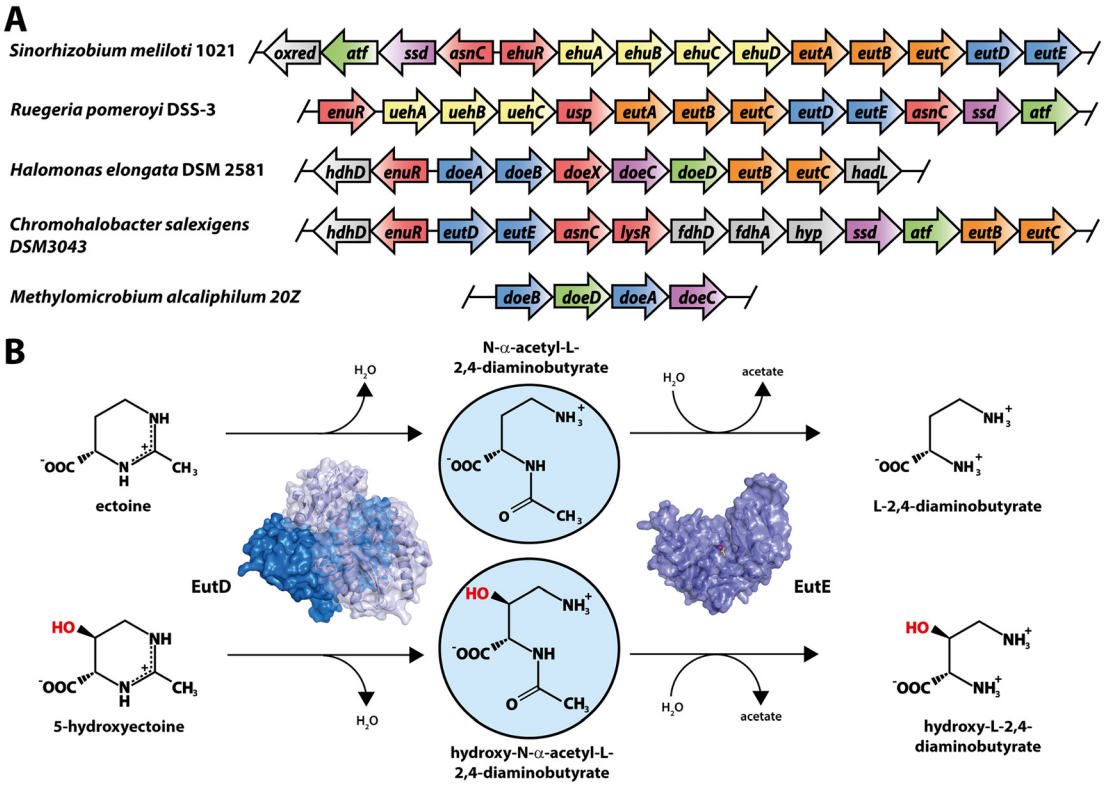

Figure 7: Genetic organization and structural enzymology of ectoine catabolism. (A) Schematic illustration of the ectoine catabolic gene clusters from *S. meliloti* (Jebbar et al. 2005), *H. elongata* (Schwibbert et al. 2011), *C. salexigens* (Vargas et al. 2006), *R. pomeroyi* (Schulz et al. 2017b) and *M. alcaliphilum* (Reshetnikov et al. 2020). (B) Pathway for the catabolism of ectoine via *N*-α-acetyl-L-2,4-diaminobutyrate to L-2,4-diaminobutyrate and for the degradation of 5-hydroxyectoine via hydroxy-*N*-α-acetyl-L-2,4-diaminobutyrat to hydroxy-L-2,4-diaminobutyrate. EutD: ectoine/5-hydroxyectoine hydrolase; EutE: *N*-α-acetyl-L-2,4-diaminobutyrate/hydroxy-*N*-α-acetyl-L-2,4-diaminobutyrat deacetylase. The crystal structures of the *H. elongata* EutD protein in complex with ectoine and the Glu-255-ADABA adduct (PDB: 6TWK) was used to visualize the dimeric protein assembly. The crystal structure of the *R. pomeroyi* EutE ADABA deacetylase in complex with the reaction products DABA and acetate (PDB: 6TWM) was used to visualize the monomeric protein. Mais et al. (2020) reported all mentioned crystal structures of the EutD and EutE proteins and details on the features of these crystal structures are given in Table 2.

Bacteria that can both synthesize and catabolize ectoine (Figure 2) represent a minority (9.5%) among the analyzed microorganisms, as most can either only synthesize or only consume ectoines. A closer inspection of the phylogenetic association of those bacteria capable to produce and to consume ectoines revealed that they are primarily represented in members of the Oceanospirillales, Vibrionales, and Rhodobacteriales (Supplementary Figure S1). Hence, a substantial fraction (41%) of bacteria predicted to produce and consume ectoines, live in marine habitats.

Genetics of ectoine and 5-hydroxyectoine degradation

An inroad into a molecular understanding of ectoine catabolism was provided by a pioneering proteomics study conducted by Jebbar et al. (2005) focusing on ectoine-inducible proteins from the plant roots-associated bacterium *Sinorhizobium meliloti* (Jebbar et al. 2005). These data promoted the identification of orthologous genes in other ectoine-consuming microorganisms; e.g., *H. elongata*, *C. salexigens*, *R. pomeroyi*, and *M. alcaliphilum* (Reshetnikov et al. 2020; Schulz et al., 2017b; Schwibbert et al. 2011; Vargas et al. 2006). In contrast to the core ectoine biosynthetic genes (*ectABC*) that are mainly found in an evolutionarily conserved operon (Czech et al. 2018a), those encoding ectoine/5-hydroxyectoine catabolic genes are highly variable with respect to both gene order and content (Jebbar et al. 2005; Reshetnikov et al. 2020; Schulz et al. 2017b; Schwibbert et al. 2011; Vargas et al. 2006) (Figure 7A).

The following basic information can be gleaned from the inspection of the various catabolic gene clusters: (i) Most of these clusters are rather large, while that from *M. alcaliphilum* seems to represent a minimal catabolic module (Reshetnikov et al. 2020). For the large gene cluster

from *R. pomeroyi*, co-transcription of the various genes (13.5 kbp) has been demonstrated (Schulz et al. 2017b); (ii) Not even the gene clusters from the phylogenetically very closely related species *H. elongata* and *C. salexigens* are identical (Copeland et al. 2011; Schwibbert et al. 2011); (iii) Some of the four large gene clusters contain genes not present in all of them; (iv) Only the gene clusters from *S. meliloti* and *R. pomeroyi* contain genes for ectoine/5-hydroxyectoine-specific transporters but the two encoded systems belong to different transporter families (Hanekop et al. 2007; Lecher et al. 2009); (v) The number and types of regulatory proteins differ between the various gene clusters (Figure 6A).

Structural enzymology of ectoine and 5-hydroxyectoine degradation

Concerted ectoine degradation by the EutD/EutE hydrolase-deacetylase complex

Organisms able to degrade ectoine, employ two enzymes, the ectoine hydrolase EutD and the *N*-acetyl-L-2,4-diaminobutyrate deacetylase EutE (Mais et al. 2020) [also referred to as DoeA and DoeB, respectively (Schwibbert et al. 2011)] (Figure 7B). These two enzymes degrade ectoine/5-hydroxyectoine into acetate and diaminobutyric acid (DABA). The resulting EutD/EutE enzyme reaction product DABA can then be further metabolized to L-aspartate by the Atf aminotransferase and the Ssd dehydrogenase (Schulz et al. 2017b; Schwibbert et al. 2011) (Figure 4). Importantly, α-ADABA, but not the major EctC substrate γ-ADABA, is the central intermediate of the concerted reactions of the ectoine hydrolase EutD and the deacetylase EutE (Mais et al. 2020). This is physiologically important, as α-ADABA, but not γ-ADABA, serves as internal inducer for ectoine/5-hydroxyectoine catabolic gene clusters regulated by the widely distributed EnuR repressor protein (Schulz et al. 2017a).

In the following sections, we summarize our structural and biochemical understanding of the enzymes making-up the pathway for the degradation of ectoines (Figure 4, *right side*).

The ectoine hydrolase: EutD

EutD (EC 3.5.4.44) reversibly catalyzes the initial step of ectoine degradation, which is the opening of the heteropyrimidine ring (Figure 7B). High-resolution crystal structures of the apo, substrate- and product-bound states of the EutD protein from *H. elongata* (*He*EutD) have been solved (Mais et al. 2020) (Table 2). These structures show that EutD forms a highly intertwined homodimer (Figure 7B) with both active sites being approximately 30 Å apart from each other (Mais et al. 2020) (Supplementary Figure S7). Each EutD monomer can be subdivided into a α-helical N-terminal domain and a C-terminal domain exhibiting a typical pita-bread fold (Bazan et al. 1994), consisting of two antiparallel β-sheets. The active site of EutD is accommodated in the center of these β-sheets, with the N-terminal domain of the opposing chain forming a lid on top of the active site. This pita-bread fold is well known from the related protein family of M24 amino-peptidases, whose enzyme activity typically depends on two highly coordinated metals (Rawlings and Barrett 1993). However, in the case of the EutD hydrolase, no metals are incorporated in the active site, as residues not suitable for metal ion coordination have replaced most of those residues typically involved in metal-coordination in M24-type amino-peptidases (Rawlings and Barrett 1993). Instead, the active site of EutD has evolved to specifically accommodate the cyclic substrate ectoine (Mais et al. 2020) (Figure 8A).

Structural analysis of the EutD homodimer in the presence of its ectoine substrate showed the ectoine substrate in one active site and the α-ADABA product covalently bound to a conserved glutamate in the other (Figure 8A,B). In the ectoine-bound active site, a water molecule hydrogen-bonded to His-238, can be found in a distance of 4 Å to the methyl group-bearing carbon of ectoine (Figure 8A). This water molecule, activated by its coordinating histidine residue might perform a nucleophilic attack on the ectoine molecule, leading to the cleavage of the heteropyrimidine ring. The EutD-mediated ring cleavage results in α-ADABA, which is covalently linked to the carboxyl side chain of Glu-255 in the other active site of the EutD homodimer. Moreover, the Glu-255-α-ADABA adduct is stabilized in the EutD active site by interactions with His-238 and Asp-338 in the active site of monomer_II (Figure 8B). Thus, structural analysis supported by mutational analysis visualized the catalytic mechanism by which EutD cleaves the ectoines ring resulting in the formation of an unusual covalent EutD-α-ADABA intermediate (Figure 8B).

The structural analysis of the ectoine hydrolase also shows that both active sites within the EutD homodimer signal their catalytic progress to each other. This communication is enabled by Tyr-52 at the N-terminus of one monomer, which protrudes into the active site of the other (and *vice versa*) to sense the catalytic state of the partnering

Table 2: Structures of ectoine and 5-hydroxyectoine degrading enzymes.

Enzyme (EC number)	Ligand	PDB accession	Resolution	Organism	References
EutD (3.5.4.44)	apo	6TWJ	2.2 Å	*H. elongata*	(Mais et al. 2020)
	ectoine; Glu255-ADABA	6TWK	2.3 Å	*H. elongata*	(Mais et al. 2020)
	N-α-ADABA; Glu255-ADABA	6YO9	2.4 Å	*H. elongata*	(Mais et al. 2020)
EutE (3.5.1.125)	apo	6TWL	2.0 Å	*R. pomeroyi*	(Mais et al. 2020)
	DABA, acetate	6TWM	2.5 Å	*R. pomeroyi*	(Mais et al. 2020)

The abbreviations used are: *N*-α-acetyl-L-2,4-diaminobutyric acid (*N*-α-ADABA); L-2,4-diaminobutyric acid (DABA).

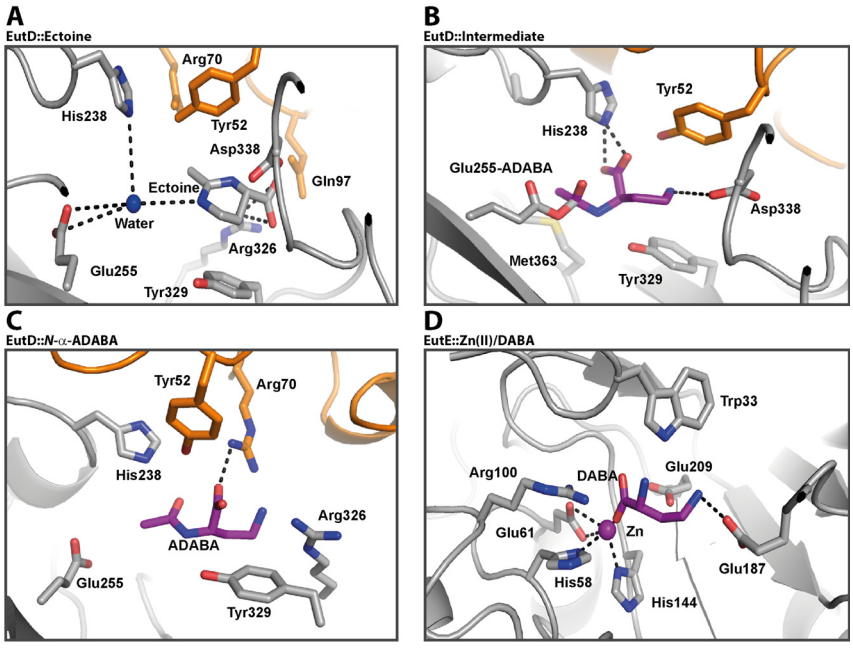

Figure 8: Structural views into the active sites of ectoine/5-hydroxyectoine degrading enzymes. (A) Architecture of the *H. elongata* ectoine/5-hydroxyectoine hydrolase (EutD). The active site_I of the dimeric EutD enzyme is shown in complex with ectoine. EutD accommodates a catalytically active water molecule by hydrogen bonds to His-238. The substrate ectoine is kept in place by hydrogen bonds to Arg-326 (PDB: 6TWK). (B) A view into the active site_II of the *H. elongata* EutD protein subsequent to ectoine hydrolysis and formation of the Glu-255-ADABA adduct. This covalent intermediate is tethered via an orthoester-like bond to the amino acid side chain of Glu-255 (PDB: 6TWK). (C) In the backward reaction of the *H. elongata* EutD enzyme (α-ADABA was provided during crystallization), the Glu-255-ADABA adduct is found in one active site of the dimer (not shown), and the second active site contains a free ADABA molecule. ADABA is coordinated by hydrogen bonds to Arg-70 (PDB: 6YO9). (D) Architecture of the *R. pomeroyi* ADABA deacetylase (EutE) active site in complex with the reaction product DABA. Crystals of EutE were grown in the presence of the EutD reaction product α-ADABA. The active site of EutE harbors a zinc-ion bound to Glu-61, His-58 and His-144. DABA coordinates to the zinc metal and hydrogen bonds to Glu-178 (PDB: 6TWM). Mais et al. (2020) reported the crystal structures of the EutD and EutE proteins.

monomer in the EutD homodimer assembly (Figure 8A–C). Superimposition of the two active sites of the EutD homodimer revealed a noticeable movement of the loop harboring Tyr-52. This movement of the "signaling loop", in turn, leads to other conformational changes in the N-terminal domain of the ectoine hydrolase (Mais et al. 2020). It can thus be imagined that the way in which the two EutD catalytic sites communicate through structural changes will aid cooperativity during the catalytic cycle (Figure 8A–C). The critical role of Tyr-52 for the communication of the two active sites in the EutD homodimer is supported by the finding that substituting Tyr-52 by alanine abolishes enzymatic activity of the ectoine hydrolase (Mais et al. 2020).

The crystal structure of EutD obtained in the presence of its reaction product α-ADABA (Figure 4) showed the same Glu-255-α-ADABA adduct observed when ectoine was supplied as the substrate (Figure 8B), while the other active site contained a free α-ADABA molecule (Figure 8C). In this crystal structure, α-ADABA is primarily hydrogen-bonded to Arg-70 from the N-terminal domain of the opposing EutD chain (Mais et al. 2020). Collectively, these two structures further supported the reversible character of the EutD-mediated cleavage of ectoine. Reversibility of the EutD-mediated reaction seems to be the reason why efficient ectoine degradation can only occur in the presence of EutE, which deacetylates α-ADABA irreversibly.

The *N*-acetyl-L-2,4-diaminobutyrate deacetylase: EutE

The *N*-acetyl-L-2,4-diaminobutyrate deacetylase EutE (EC 3.5.1.125) converts α-ADABA into DABA (Mais et al. 2020;

Schulz et al. 2017b; Schwibbert et al. 2011) (Figure 4). Biochemical analysis of the *R. pomeroyi* EutE enzyme demonstrated that EutE exclusively processes *N*-α-ADABA in an highly efficient manner, while its isomer γ-ADABA, the substrate of the ectoine synthase (Czech et al. 2019a), cannot be deacetylated (Mais et al. 2020) (Figure 8C) (Supplementary Figure S8). These data are consistent with reports on the properties of the corresponding *H. elongata* enzyme (Schwibbert et al. 2011).

Crystal structures of EutE from *R. pomeroyi* are available in the apo state and bound to its products DABA and acetate (Table 2) (Mais et al. 2020), revealing structural homology to asparto-acylases (Le Coq et al. 2008). Conserved features among these types of enzymes are the crescent-like shape with two distinct subdomains (Figure 7B) and a zinc-binding site formed one Glu and two His residues (Figure 8D) (Mais et al. 2020). The products of *N*-α-ADABA deacetylation, DABA and acetate, both coordinate the central zinc-atom in the active site of the EutE enzyme with their carboxyl moieties. Comparison of the EutE crystal structure to asparto-acylases, which have been crystalized in presence of a substrate-mimic (Le Coq et al. 2008), as well as mutational analysis of EutE, hint that Arg-111 is most likely hydrogen bonding the second carboxyl moiety of *N*-α-ADABA, while Asp-195 hydrogen bonds the amine group, thus fixating the substrate in the enzyme reaction chamber. During the hydrolysis of *N*-α-ADABA, which proceeds via a tetrahedral transition state, the guanidinium moiety of Arg-100 (Figure 8D) further compensates the emerging negative charge (Mais et al. 2020).

We would like to note that EutE forms stable hexamers in solution when substrates, products and the catalytically essential zinc ion are absent (Mais et al. 2020). However, our crystallographic analysis did not support such a ternary assembly as all crystallographic contacts appear of non-biologically relevant nature. Therefore, we suggested that the active form of EutE is a monomer (Mais et al. 2020). Whether the observed EutE hexamer in solution is of functional relevance or simply a concentration artifact remains to be determined.

Heterologous production of the EutD hydrolase from *R. pomeroyi* and *M. alcaliphilum* in *E. coli* in the presence of ectoine yielded exclusively *N*-α-ADABA and, when the *Rp*EutD enzyme was exposed *in vivo* to 5-hydroxyectoine, also hydroxy-*N*-α-ADABA (Mais et al. 2020; Reshetnikov et al. 2020). In contrast, the heterologous production of the EutD (DoeA) hydrolase from *H. elongata* seemingly yielded both α- and γ-ADABA, with the apparent use of *N*-γ-ADABA for a new round of ectoine synthesis via EctC in *H. elongata* (Schwibbert et al. 2011). Hence, the catalytic profile of the ectoine hydrolase from *M. alcaliphilum* and *H. elongata* seem to differ, despite the fact that both extremophiles can consume and produce ectoine (Reshetnikov et al. 2020; Schwibbert et al., 2011).

Topology of the ectoine/5-hydroxyectoine catabolic route

In a study that specifically addressed the catabolism of 5-hydroxyectoine, Schulz et al. (2017) suggested that the use of this compound as nutrient would require the initial removal of the hydroxyl group by the EutABC enzymes to form ectoine (Schulz et al. 2017a,b). Consistent with this hypothesis was the finding that an *eutABC* deletion of the *R. pomeroyi* catabolic gene cluster abolished the use of 5-hydroxyectoine but not that of ectoine (Schulz et al. 2017b). However, the finding of Mais et al. (2020) that the *Rp*EutD hydrolase can also open the heteropyrimidine ring of 5-hydroxyectoine to form hydroxy-*N*-α-ADABA (Figure 8B) requires a re-interpretation of the data reported by Schulz et al. (2017). Rather than being initially involved in removing the hydroxyl group from the 5-hydroxyectoine ring, the growth defect of the *R. pomeroyi eutABC* deletion mutant on 5-hydroxyectoine as the carbon source suggests now a role of either all, or at least some, of the EutABC proteins in the down-stream metabolic procession of the EutD- or EutD/EutE-formed metabolites (Figure 4).

The EutABC enzymes are currently annotated in the *R. pomeroyi* genome sequence as Asp/Glu/hydantoine racemase (EutA), as L-threonine ammonia lyase (EutB), and as ornithine cyclodeaminase (EutC) (Moran et al. 2004). Hence, the EutB/EutC enzymes might contribute at the level of the hydroxy-*N*-α-ADABA intermediate to the catabolism of 5-hydroxyectoine (Figure 4). EutB shares significant homology to the PLP-dependent group of threonine dehydratases. Hence, it seems possible that EutB removes the hydroxyl moiety from hydroxy-*N*-α-ADABA, and this novel intermediate could then be reduced by EutC in a NADPH-dependent manner to yield *N*-α-ADABA. *N*-α-ADABA would then serve as a *bona-fide* substrate for the EutE deacetylase (Figure 4). Interestingly, a pair of functionally related enzymes (BhcB and BhcD) operates in a similar manner in the widely distributed β-hydroxyaspartate pathway of microorganisms (Schada von Borzyskowski et al. 2019). The role of EutA in such a scheme remains uncertain and different entry points of the EutABC enzymes into the overall 5-hydroxyectoine catabolic rout remain a possibility (Figure 4). Notably, there exist a considerable number of ectoine/5-hydroxyectoine catabolic gene clusters that lack *eutA* (Mais et al. 2020; Schulz

et al. 2017b), indicating the ectoine/5-hydroxyectoine catabolic route might not be identical in microorganisms consuming ectoines.

Genetic regulation of ectoine metabolism - lessons from structural enzymology?

Reflecting the major function of ectoines as osmostress protectants, the transcription of *ect* biosynthetic gene clusters is typically strongly up-regulated when cells experience either sudden or sustained high osmolarity (Czech et al., 2018a; Kunte et al. 2014; Pastor et al. 2010). However, the molecular underpinnings of the signal transduction process that emanates from the increase in the environmental osmolarity and eventually determines the transcriptional activity of promoters for ectoine biosynthetic genes has largely remained enigmatic [for references and a critical discussion of the literature related this issue see (Czech et al. 2018a)].

Data from the detailed molecular analysis of the *ect* promoter from the plant roots-associated bacterium *P. stutzeri* A1501 are however noteworthy in this context (Czech et al. 2018b). Transcriptional activity, and hence ectoine production, of this *ect* promoter is linearly dependent on the degree of the imposed osmotic stress, implying that the cell can detect and react to incremental increases in environmental osmolarity. This transcription pattern of the *P. stutzeri ect* promoter was fully replicated in the non ectoine producing surrogate host bacterium *E. coli*. Furthermore, osmotic induction was still preserved when the G/C-rich -10 region of the *ect* promoter was mutated to a perfect sigma-70-type promoter that is typically A/T-rich (Czech et al. 2018b). Taken together, these data suggest that osmotic regulation seems to be an inherent feature of the promoter elements, the spacer and flanking regions.

However, in some organisms regulatory proteins have been shown to be involved in the regulation of the ectoine biosynthetic genes, such as the MarR-type regulators EctR from *M. alcaliphilum* (Mustakhimov et al. 2010) or CosR from *Vibrio cholera* (Gregory et al. 2019, 2020; Shikuma et al. 2013). Interestingly, for CosR ionic strength has been proposed as the key factor determining its DNA-binding activity (Shikuma et al. 2013). In the case of EctR, its genetic inactivation increased *ect* transcription notably but osmoregulation of this gene cluster was maintained (Mustakhimov et al. 2010). However, it remains unknown, which factors trigger EctR binding and release from the DNA. In *Streptomyces coelicolor* the global regulator for nitrogen metabolism, GlnR, also serves as a repressor for the expression of the ectoine/5-hydroxyectoine biosynthetic genes (Shao et al. 2015). This finding, links the genetic control of nitrogen metabolism in *Streptomycetes* with the regulation of a gene cluster encoding the enzymes for the synthesis of the nitrogen-rich ectoines (Bursy et al. 2008; Kol et al. 2010).

In contrast to osmotic control the biosynthetic gene clusters (Czech et al. 2018a), expression of the catabolic gene clusters [at least for the two that have been studied in more detail (e.g., *S. meliloti* and *R. pomeroyi*)], is substrate inducible. Their transcription is strongly up-regulated when ectoines are present in the growth medium (Jebbar et al. 2005; Schulz et al. 2017a; Yu et al. 2017) but ectoines are not the true inducers (see below) (Schulz et al. 2017a). Most inspected ectoine/5-hydroxyectoine catabolic gene clusters [456 out of 539 (Schulz et al. 2017a);] contain a gene encoding a member (EhuR/EnuR) of the MocR/GabR-family of transcriptional regulators (Tramonti et al. 2018). Detailed studies on the EhuR/EnuR regulatory proteins from *S. meliloti* and *R. pomeroyi* identified the degradation intermediates *N*-α-ADABA and DABA as system-specific inducers and ligands for EhuR/EnuR (Schulz et al. 2017a; Yu et al. 2017). MocR/GabR-type regulators typically consist of a N-terminal winged-helix-turn-helix DNA-reading head connected via a long and highly flexible linker to a C-terminal aminotransferase domain of fold I (Edayathumangalam et al. 2013; Tramonti et al. 2018). The aminotransferase domain does not possess enzymatic function; instead the chemistry of a covalently bound PLP in a reaction with a systems-specific low-molecular-weight effector molecule triggers a conformational change affecting DNA binding (Edayathumangalam et al. 2013; Frezzini et al. 2020; Tramonti et al. 2018; Wu et al. 2017).

In EnuR, the PLP cofactor is covalently bound to Lys-302 forming an internal aldimine. The formation of the intermediate of ectoine degradation *N*-α-ADABA in cells catabolizing ectoine results in the binding of *N*-α-ADABA, or DABA, to PLP thereby forming an external aldimine (Schulz et al. 2017a). These chemical reactions trigger a conformational change of the EnuR regulator, and concurrently relieves the transcriptional repression of the ectoine/5-hydroxyectoine catabolic gene cluster. The replacement of Lys-302 with an amino acid residue to which PLP cannot be covalently attached transforms the mutant EnuR protein into a super-repressor that no longer responds to its ectoine-derived internal inducers (Schulz et al. 2017a). Physiologically important is the fact that in contrast to the specific ectoine metabolite *N*-α-ADABA, *N*-γ-ADABA, the major substrate of the ectoine synthase EctC (Czech et al. 2019a), does not serve as an inducer of the catabolic genes (Schulz et al. 2017a).

It has recently been found that the hydroxylated forms of *N*-α-ADABA and DABA are selectively generated during the EutD-mediated hydrolysis of 5-hydroxyectoine (Mais et al. 2020) (Figure 4). Because external 5-hydroxyectoine is a strong inducer of the *R. pomeroyi* ectoine/5-hydroxyectoine catabolic genes (Schulz et al. 2017a), it seems plausible that hydroxy-*N*-α-ADABA and hydroxy-DABA will also serve as internal inducers for the EnuR repressor.

Many ectoine/5-hydroxyectoine catabolic gene clusters also contain a gene (*asnC*/*doeX*) (Figure 7A) for a member of the feast and famine family of transcriptional regulators (Schulz et al. 2017a; Schwibbert et al. 2011; Yokoyama et al. 2006). These types of proteins can wrap DNA into nucleosome-like structures and frequently respond in their DNA-binding activity to low-molecular weight effector molecules (e.g., amino acids) (Dey et al. 2016; Kumarevel et al. 2008; Shrivastava and Ramachandran 2007). Schwibbert et al. (2011) showed that such an AsnC-type protein (referred to by these authors as DeoX) targets DNA sequences located at or in the vicinity of the promoter for the *H. elongata* ectoine/5-hydroxyectoine catabolic gene cluster (Schwibbert et al. 2011). Notably, AsnC serves as an activator for the *R. pomeroyi* import and catabolic gene cluster and its loss abolished the use of ectoine as sole carbon but not as sole nitrogen source (Schulz et al. 2017a). Details of the structure and genetic mode of action of the DoeX- and AsnC-type proteins still need to be worked out and their effector molecule(s) must be identified. An educated guess would suggest that these regulatory proteins would bind either ectoines or metabolites derived from them as effectors.

Perspectives and open questions

Marking the 35th anniversary of the seminal discovery of the major microbial cytoprotectant ectoine (Galinski et al. 1985) and the detection of its derivative 5-hydroxyectoine just a few years later (Inbar and Lapidot 1988), a complete structural view and an in-depth biochemical understanding of the ectoine/5-hydroxyectoine biosynthetic route is now available (Figure 5). Not only are ectoines excellent cytoprotectants (Czech et al. 2018a; Pastor et al. 2010) but these natural products are also of considerable commercial value (Becker and Wittmann 2020; Kunte et al. 2014). Innovative uses of laboratory evolution experiments, site-directed mutagenesis, exploits of ectoine-responsive biosensors (Chen et al. 2015), and synthetic microbiology approaches (Giesselmann et al. 2019) should aid further improvements in the productivity of synthetic or natural cell factories for ectoine and 5-hydroxyectoine. The changes resulting from such experiments can then be correlated to specific changes in either regulatory DNA sequences or features of the ectoine/5-hydroxyectoine biosynthetic enzymes, to further illuminate the salient genetic and biochemical features of the ectoine/5-hydroxyectoine biosynthetic route required for the optimal synthesis of these commercially valuable compounds.

The actual mechanism of osmosensing and the molecular biology allowing osmotically stressed microbial cells to transform this environmental cue into enhanced promoter activity of ectoine biosynthetic genes is only rudimentarily comprehended. Is osmotic control of *ect* transcription mediated by specific regulatory proteins, or is this an intrinsic property of the given promoter and its flanking region? Contributors to this latter regulatory modus might be changes in DNA-topology under osmotic stress conditions (Higgins et al. 1988) and altered interaction of RNA-polymerase with -10 and -35 promoter regions in a crowded cytoplasm with changing solvation attributes when the external osmolarity is raised (Cagliero and Jin 2012; Cayley et al. 1991; Gralla and Huo 2008; Record et al. 1998; van den Berg et al. 2017). The mode of action of transcriptional regulators (e.g., EctR, CosR, GlnR, AphA, OpaR, EupR, and alternative sigma factors) that have been implicated in the control of *ect* promoter activity in various bacteria certainly deserve future study and scrutiny (Calderon et al. 2004; Gregory et al. 2019, 2020; Kuhlmann et al. 2011; Mustakhimov et al. 2010; Rodriguez-Moya et al. 2010; Schwibbert et al. 2011; Shao et al. 2015; Shikuma et al. 2013).

Biosynthesis and the ecophysiology of ectoines have been actively studied for a considerable time but the uses of these cytoprotectants as nutrients have only come recently into focus (Jebbar et al. 2005; Reshetnikov et al. 2020; Schulz et al. 2017b; Schwibbert et al. 2011; Vargas et al. 2006). A major advance has now been achieved through the structural analysis of the EutD/EutE hydrolase/deacetylase enzyme bi-module that opens the heteropyrimidine ring of ectoine and 5-hydroxyectoine and processes the formed *N*-α-ADABA and hydroxy-*N*-α-ADABA for further catabolism (Mais et al. 2020) (Figures 4 and 7B). The intermediate *N*-α-ADABA, and presumably also hydroxy-*N*-α-ADABA, serve as high-affinity internal inducers for the EnuR repressor, thereby triggering substrate-mediated induction of ectoine/5-hydroxectoine catabolic gene clusters (Schulz et al. 2017a). Further molecular and biochemical studies should now advance our understanding of this interesting PLP-dependent MocR/GabR-type repressor (Tramonti et al. 2018).

While the recent biochemical and structural analysis of the ectoine/5-hydroxyectoine hydrolase (EutD) and the *N*-α-ADABA-deacetylase (EutE) is a major advance, important questions remain with respect to those enzymes (EutABC) operating down-stream of the EutD/EutE enzyme bi-module (Figure 4). The elucidation of the function of these enzymes, and hence the clarification of the topology of the ectoine/5-hydroxyectoine degradation pathway as a whole, requires a concerted biochemical and physiological approach that needs to be supported by studies using well defined single gene deletion mutant strains. The synthesis and degradation routes of ectoines can be viewed as opposing pathways; yet they share a number of common intermediates (Figure 4). While the ability to synthesize or consume ectoines is clearly separated in most bacteria (Supplementary Figure S1), there is an interesting group of microorganisms that can do both (Reshetnikov et al. 2020; Schwibbert et al. 2011; Vargas et al. 2006). These latter organisms are faced with a potentially wasteful futile cycle. How does the cell balance the two physiological tasks at hand, osmostress protection and nutrient utilization, under osmotic steady-state growth conditions? Can it take a physiological advantage from a continuously running synthesis - degradation process (Reshetnikov et al. 2020; Schwibbert et al. 2011)?

The ecophysiological importance of ectoines for microbial assemblages and biofilms has only recently gained increased attention. For instance, transcription of the ectoine biosynthetic genes and corresponding transport systems were strongly induced when biofilms of *Sphingomonas* sp. LH128 (reclassified as *Novosphingobium* sp.) were subjected to an osmotic up-shock (Fida et al. 2012). This finding points to a role of ectoines in protecting cells encased in biofilms, multi-cellular assemblages strained and architecturally shaped by osmotic forces (Rubinstein et al. 2012; Seminara et al. 2012; Yan et al. 2017). In the pathogens *Vibrio cholerae* and *Vibrio parahaemolyticus* (and taxonomically related species) the genetic control of ectoine biosynthesis, compatible solute import, and biofilm formation is embedded in complex regulatory circuits that also involve quorum-sensing-type transcriptional regulators (Gregory et al. 2019, 2020; Shikuma et al. 2013). Dissecting these regulatory processes will open exciting entire new avenues for research. Released/actively secreted ectoines might also become a public good for microbial communities, as has been found for glycine betaine in the case of *V. cholerae* (Kapfhammer et al. 2005).

The unexpected discovery of ectoine/5-hydroxy ectoine-producing ciliates and micro-algae and the way in which these eukaryotic cells interact with bacteria paves the way for studies addressing the role of these cytoprotectants in the framework of microbial ecology (Fenizia et al. 2020; Harding et al. 2016; Landa et al. 2017; Weinisch et al. 2019). It is of interest to note in this context that the plant roots-associated bacterium *S. meliloti* performs chemotaxis towards the plant-produced compatible solute proline betaine and other types of quaternary ammonium compounds (e.g., glycine betaine) found in root exudates (Shrestha et al. 2018; Webb et al. 2017). Hence, one might ask if environmental ectoines might serve as chemo-attractants when microorganisms seek them out either as stress protectants or nutrients.

And finally, what are the molecular and physicochemical mechanisms when ectoines are used either as cold- or heat-stress protectants (Bursy et al. 2008; Garcia-Estepa et al. 2006; Kuhlmann et al. 2008a, 2011; Ma et al. 2017)? Are the principles underlying thermoprotection by ectoines different from those that govern their function as osmostress protectants, or are there mechanistically unifying underpinnings?

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References

Aik, W., McDonough, M.A., Thalhammer, A., Chowdhury, R., and Schofield, C.J. (2012). Role of the jelly-roll fold in substrate binding by 2-oxoglutarate oxygenases. Cur. Opin. Struc. Biol. 22: 691–700.

Arakawa, T., and Timasheff, S.N. (1985). The stabilization of proteins by osmolytes. Biophys. J. 47: 411–414.

Barth, S., Huhn, M., Matthey, B., Klimka, A., Galinski, E.A., and Engert, A. (2000). Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. Appl. Environ. Microbiol. 66: 1572–1579.

Bayles, D.O., Tunick, M.H., Foglia, T.A., and Miller, A.J. (2000). Cold shock and its effect on ribosomes and thermal tolerance in *Listeria monocytogenes*. Appl. Environ. Microbiol. 66: 4351–4355.

Bazan, J.F., Weaver, L.H., Roderick, S.L., Huber, R., and Matthews, B.W. (1994). Sequence and structure comparison suggest that methionine aminopeptidase, prolidase, aminopeptidase P, and creatinase share a common fold. Proc. Natl. Acad. Sci. U.S.A. 91: 2473–2477.

Becker, J., Schäfer, R., Kohlstedt, M., Harder, B.J., Borchert, N.S., Stöveken, N., Bremer, E., and Wittmann, C. (2013). Systems metabolic engineering of *Corynebacterium glutamicum* for production of the chemical chaperone ectoine. Microb. Cell Fact. 12: 110.

Becker, J., and Wittmann, C. (2020). Microbial production of extremolytes - high-value active ingredients for nutrition, health care, and well-being. Curr. Opin. Biotechnol. 65: 118–128.

Bestvater, T., Louis, P., and Galinski, E.A. (2008). Heterologous ectoine production in *Escherichia coli:* by-passing the metabolic bottle-neck. Saline Syst. 4: 12.

Biran, D., Rotem, O., Rosen, R., and Ron, E.Z. (2018). Coping with high temperature: a unique regulation in *A. tumefaciens*. Curr. Top. Microbiol. Immunol. 418: 185–194.

Bolen, D.W., and Baskakov, I.V. (2001). The osmophobic effect: natural selection of a thermodynamic force in protein folding. J. Mol. Biol. 310: 955–963.

Booth, I.R. (2014). Bacterial mechanosensitive channels: progress towards an understanding of their roles in cell physiology. Curr. Opin. Microbiol. 18: 16–22.

Bourot, S., Sire, O., Trautwetter, A., Touze, T., Wu, L.F., Blanco, C., and Bernard, T. (2000). Glycine betaine-assisted protein folding in a *lysA* mutant of *Escherichia coli*. J. Biol. Chem. 275: 1050–1056.

Bouskill, N.J., Wood, T.E., Baran, R., Ye, Z., Bowen, B.P., Lim, H., Zhou, J., Nostrand, J.D., Nico, P., Northen, T.R., et al. (2016). Belowground response to drought in a tropical forest soil. I. changes in microbial functional potential and metabolism. Front. Microbiol. 7: 525.

Bremer, E., and Krämer, R. (2019). Responses of microorganisms to osmotic stress. Annu. Rev. Microbiol. 73: 313–314.

Brown, A.D. (1976). Microbial water stress. Bacteriol. Rev. 40: 803–846.

Bruce, H., Nguyen Tuan, A., Mangas Sanchez, J., Leese, C., Hopwood, J., Hyde, R., Hart, S., Turkenburg, J.P., and Grogan, G. (2012). Structures of a γ-aminobutyrate (GABA) transaminase from the s-triazine-degrading organism *Arthrobacter aurescens* TC1 in complex with PLP and with its external aldimine PLP-GABA adduct. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 68: 1175–1180.

Bursy, J., Kuhlmann, A.U., Pittelkow, M., Hartmann, H., Jebbar, M., Pierik, A.J., and Bremer, E. (2008). 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. 74: 7286–7296.

Bursy, J., Pierik, A.J., Pica, N., and Bremer, E. (2007). Osmotically induced synthesis of the compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine hydroxylase. J. Biol. Chem. 282: 31147–31155.

Cagliero, C., and Jin, D.J. (2012). Dissociation and re-association of RNA polymerase with DNA during osmotic stress response in *Escherichia coli*. Nucleic Acids Res. 41: 315–326.

Caldas, T., Demont-Caulet, N., Ghazi, A., and Richarme, G. (1999). Thermoprotection by glycine betaine and choline. Microbiology 145: 2543–2548.

Calderon, M.I., Vargas, C., Rojo, F., Iglesias-Guerra, F., Csonka, L.N., Ventosa, A., and Nieto, J.J. (2004). Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043T. Microbiology 150: 3051–3063.

Canovas, D., Borges, N., Vargas, C., Ventosa, A., Nieto, J.J., and Santos, H. (1999). Role of *N*-γ-acetyldiaminobutyrate as an enzyme stabilizer and an intermediate in the biosynthesis of hydroxyectoine. Appl. Environ. Microbiol. 65: 3774–3779.

Capp, M.W., Pegram, L.M., Saecker, R.M., Kratz, M., Riccardi, D., Wendorff, T., Cannon, J.G., and Record, M.T., Jr. (2009). Interactions of the osmolyte glycine betaine with molecular surfaces in water: thermodynamics, structural interpretation, and prediction of m-values. Biochemistry 48: 10372–10379.

Cayley, S., Lewis, B.A., Guttman, H.J., and Record, M.T., , Jr. (1991). Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein-DNA interactions *in vivo*. J. Mol. Biol. 222: 281–300.

Cayley, S., and Record, M.T. , Jr. (2003). Roles of cytoplasmic osmolytes, water, and crowding in the response of *Escherichia coli* to osmotic stress: biophysical basis of osmoprotection by glycine betaine. Biochemistry 42: 12596–12609.

Chattopadhyay, M.K., Kern, R., Mistou, M.Y., Dandekar, A.M., Uratsu, S.L., and Richarme, G. (2004). The chemical chaperone proline relieves the thermosensitivity of a *dnaK* deletion mutant at 42 degrees C. J. Bacteriol. 186: 8149–8152.

Chen, I.A., Chu, K., Palaniappan, K., Pillay, M., Ratner, A., Huang, J., Huntemann, M., Varghese, N., White, J.R., Seshadri, R., et al. (2019). IMG/M v.5.0: an integrated data management and comparative analysis system for microbial genomes and microbiomes. Nucleic Acids Res. 47: D666–D677.

Chen, W., Zhang, S., Jiang, P.X., Yao, J., He, Y.Z., Chen, L.C., Gui, X.W., Dong, Z.Y., and Tang, S.Y. (2015). Design of an ectoine-responsive AraC mutant and its application in metabolic engineering of ectoine biosynthesis. Metab. Eng. 30: 149–155.

Copeland, A., O'Connor, K., Lucas, S., Lapidus, A., Berry, K.W., Detter, J.C., Del Rio, T.G., Hammon, N., Dalin, E., Tice, H., et al. (2011). Complete genome sequence of the halophilic and highly halotolerant *Chromohalobacter salexigens* type strain (1H11(T)). Stand. Genomic Sci. 5: 379–388.

Czech, L., Hermann, L., Stöveken, N., Richter, A.A., Höppner, A., Smits, S.H.J., Heider, J., and Bremer, E. (2018a). Role of the extremolytes ectoine and hydroxyectoine as stress protectants and nutrients: genetics, phylogenomics, biochemistry, and structural analysis. Genes 9: 177.

Czech, L., Höppner, A., Kobus, S., Seubert, A., Riclea, R., Dickschat, J.S., Heider, J., Smits, S.H.J., and Bremer, E. (2019a). Illuminating the catalytic core of ectoine synthase through structural and biochemical analysis. Sci. Rep. 9: 364.

Czech, L., Poehl, S., Hub, P., Stoeveken, N., and Bremer, E. (2018b). Tinkering with osmotically controlled transcription allows enhanced production and excretion of ectoine and

hydroxyectoine from a microbial cell factory. Appl. Environ. Microbiol. 84: e01772-17.

Czech, L., Stöveken, N., and Bremer, E. (2016). EctD-mediated biotransformation of the chemical chaperone ectoine into hydroxyectoine and its mechanosensitive channel-independent excretion. Microb. Cell Fact. 15: 126.

Czech, L., Wilcken, S., Czech, O., Linne, U., Brauner, J., Smits, S.H.J., Galinski, E.A., and Bremer, E. (2019b). Exploiting substrate promiscuity of ectoine hydroxylase for regio- and stereoselective modification of homoectoine. Front. Microbiol. 10: 2745.

D'Ari, R., and Casadesus, J. (1998). Underground metabolism. Bioessays 20: 181–186.

da Costa, M.S., Santos, H., and Galinski, E.A. (1998). An overview of the role and diversity of compatible solutes in *Bacteria* and *Archaea*. Adv. Biochem. Eng. Biotechnol. 61: 117–153.

Danchin, A., and Nikel, P.I. (2019). Why nature chose potassium. J. Mol. Evol. 87: 271–288.

Dey, A., Shree, S., Pandey, S.K., Tripathi, R.P., and Ramachandran, R. (2016). Crystal structure of *Mycobacterium tuberculosis* H37Rv AldR (rv2779c), a regulator of the *ald* gene: DNA-binding, and identification of small-molecule inhibitors. J. Biol. Chem. 291: 11967–11980.

Diamant, S., Eliahu, N., Rosenthal, D., and Goloubinoff, P. (2001). Chemical chaperones regulate molecular chaperones i*n vitro* and in cells under combined salt and heat stresses. J. Biol. Chem. 276: 39586–39591.

Dunwell, J.M., Purvis, A., and Khuri, S. (2004). Cupins: the most functionally diverse protein superfamily? Phytochemistry 65: 7–17.

Edayathumangalam, R., Wu, R., Garcia, R., Wang, Y., Wang, W., Kreinbring, C.A., Bach, A., Liao, J., Stone, T.A., Terwilliger, T.C., et al. (2013). Crystal structure of *Bacillus subtilis* GabR, an autorepressor and transcriptional activator of *gabT*. Proc. Natl. Acad. Sci. U.S.A. 110: 17820–17825.

Eiberweiser, A., Nazet, A., Kruchinin, S.E., Fedotova, M.V., and Buchner, R. (2015). Hydration and ion binding of the osmolyte ectoine. J. Phys. Chem. B 119: 15203–15211.

Fenizia, S., Thume, K., Wirgenings, M., and Pohnert, G. (2020). Ectoine from bacterial and algal origin Is a compatible solute in microalgae. Mar. Drugs 18, https://doi.org/10.3390/md18010042.

Fida, T.T., Breugelmans, P., Lavigne, R., Coronado, E., Johnson, D.R., van der Meer, J.R., Mayer, A.P., Heipieper, H.J., Hofkens, J., and Springael, D. (2012). Exposure to solute stress affects genome-wide expression but not the polycyclic aromatic hydrocarbon-degrading activity of *Sphingomonas* sp. strain LH128 in biofilms. Appl. Environ. Microbiol. 78: 8311–8320.

Frezzini, M., Narzi, D., Sciolari, A.M., Guidoni, L., and Pascarella, S. (2020). Molecular dynamics of an asymmetric form of GabR, a bacterial transcriptional regulator. Biophys. Chem. 262: 106380.

Galinski, E.A., Pfeiffer, H.P., and Trüper, H.G. (1985). 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. 149: 135–139.

Galinski, E.A., Stein, M., Ures, A., and Schwarz, T. (2009). *Stereo-specific hydroxylation* (International patent application WO 2009/059783 A1).

Galinski, E.A., and Trüper, H.G. (1994). Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol. Rev. 15: 95–108.

Garcia-Estepa, R., Argandona, M., Reina-Bueno, M., Capote, N., Iglesias-Guerra, F., Nieto, J.J., and Vargas, C. (2006). 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. 188: 3774–3784.

Giesselmann, G., Dietrich, D., Jungmann, L., Kohlstedt, M., Jeon, E.J., Yim, S.S., Sommer, F., Zimmer, D., Muhlhaus, T., Schroda, M., et al. (2019). Metabolic engineering of *Corynebacterium glutamicum* for high-level ectoine production - design, combinatorial assembly and implementation of a transcriptionally balanced heterologous ectoine pathway. Biotechnol. J. 14: e201800417.

Gralla, J.D., and Huo, Y.X. (2008). Remodeling and activation of *Escherichia coli* RNA polymerase by osmolytes. Biochemistry 47: 13189–13196.

Grammann, K., Volke, A., and Kunte, H.J. (2002). 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. 184: 3078–3085.

Gregory, G.J., Morreale, D.P., and Boyd, E.F. (2020). CosR is a global regulator of the osmotic stress response with widespread distribution among bacteria. Appl. Environ. Microbiol. 86: e00120-20. https://doi.org/10.1128/AEM.00120-20.

Gregory, G.J., Morreale, D.P., Carpenter, M.R., Kalburge, S.S., and Boyd, E.F. (2019). Quorum sensing regulators AphA and OpaR control expression of the operon responsible for biosynthesis of the compatible solute ectoine. Appl. Environ. Microbiol. 85: e01543-19.

Gunde-Cimerman, N., Plemenitas, A., and Oren, A. (2018). Strategies of adaptation of microorganisms of the three domains of life to high salt concentrations. FEMS Microbiol. Rev. 42: 353–375.

Hahn, M.B., Meyer, S., Schroter, M.A., Kunte, H.J., Solomun, T., and Sturm, H. (2017). DNA protection by ectoine from ionizing radiation: molecular mechanisms. Phys. Chem. Chem. Phys. 19: 25717–25722.

Hahn, M.B., Smales, G.J., Seitz, H., Solomun, T., and Sturm, H. (2020). Ectoine interaction with DNA: influence on ultraviolet radiation damage. Phys. Chem. Chem. Phys. 22: 6984–6992.

Hanekop, N., Höing, M., Sohn-Bösser, L., Jebbar, M., Schmitt, L., and Bremer, E. (2007). Crystal structure of the ligand-binding protein EhuB from *Sinorhizobium meliloti* reveals substrate recognition of the compatible solutes ectoine and hydroxyectoine. J. Mol. Biol. 374: 1237–1250.

Hangasky, J.A., Taabazuing, C.Y., Valliere, M.A., and Knapp, M.J. (2013). Imposing function down a (cupin)-barrel: secondary structure and metal stereochemistry in the alphaKG-dependent oxygenases. Metallomics 5: 287–301.

Hara, R., Nishikawa, T., Okuhara, T., Koketsu, K., and Kino, K. (2019). Ectoine hydroxylase displays selective trans-3-hydroxylation activity towards L-proline. Appl. Microbiol. Biotechnol. 103: 5689–5698.

Harding, T., Brown, M.W., Simpson, A.G., and Roger, A.J. (2016). Osmoadaptative strategy and its molecular signature in obligately halophilic heterotrophic protists. Genome Biol. Evol. 8: 2241–2258.

Harishchandra, R.K., Sachan, A.K., Kerth, A., Lentzen, G., Neuhaus, T., and Galla, H.J. (2011). Compatible solutes: ectoine and hydroxyectoine improve functional nanostructures in artificial lung surfactants. Biochim. Biophys. Acta 1808: 2830–2840.

Harishchandra, R.K., Wulff, S., Lentzen, G., Neuhaus, T., and Galla, H.J. (2010). The effect of compatible solute ectoines on the structural organization of lipid monolayer and bilayer membranes. Biophys. Chem. 150: 37–46.

Hengge-Aronis, R. (2002). Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. 66: 373–395.

Herr, C.Q., and Hausinger, R.P. (2018). Amazing diversity in biochemical roles of Fe(II)/2-oxoglutarate oxygenases. Trends Biochem. Sci. 43: 517–532.

Herzog, M., Dwivedi, M., Kumar Harishchandra, R., Bilstein, A., Galla, H.J., and Winter, R. (2019). Effect of ectoine, hydroxyectoine and beta-hydroxybutyrate on the temperature and pressure stability of phospholipid bilayer membranes of different complexity. Colloids Surf. B Biointerfaces 178: 404–411.

Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G., and Bremer, E. (1988). A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. Cell 52: 569–584.

Hillier, H.T., Altermark, B., and Leiros, I. (2020). The crystal structure of the tetrameric DABA-aminotransferase EctB, a rate-limiting enzyme in the ectoine biosynthesis pathway. FEBS J. in press, https://doi.org/10.1111/febs.15265.

Hoffmann, T., von Blohn, C., Stanek, A., Moses, S., Barzantny, S., and Bremer, E. (2012). Synthesis, release, and recapture of the compatible solute proline by osmotically stressed *Bacillus subtilis* cells. Appl. Environ. Microbiol. 78: 5753–5762.

Höppner, A., Widderich, N., Lenders, M., Bremer, E., and Smits, S.H.J. (2014). Crystal structure of the ectoine hydroxylase, a snapshot of the active site. J. Biol. Chem. 289: 29570–29583.

Ignatova, Z., and Gierasch, L.M. (2006). Inhibition of protein aggregation *in vitro* and *in vivo* by a natural osmoprotectant. Proc. Nat. Acad. Sci. USA. 103: 13357–13361.

Inbar, L., Frolow, F., and Lapidot, A. (1993). The conformation of new tetrahydropyrimidine derivatives in solution and in the crystal. Eur. J. Biochem. 214: 897–906.

Inbar, L., and Lapidot, A. (1988). The structure and biosynthesis of new tetrahydropyrimidine derivatives in actinomycin D producer *Streptomyces parvulus*. Use of ^{13}C- and ^{15}N-labeled L-glutamate and ^{13}C and ^{15}N NMR spectroscopy. J. Biol. Chem. 263: 16014–16022.

Islam, M.S., Leissing, T.M., Chowdhury, R., Hopkinson, R.J., and Schofield, C.J. (2018). 2-Oxoglutarate-dependent oxygenases. Annu. Rev. Biochem. 87: 585–620.

Jebbar, M., Sohn-Bösser, L., Bremer, E., Bernard, T., and Blanco, C. (2005). Ectoine-induced proteins in *Sinorhizobium meliloti* include an ectoine ABC-type transporter involved in osmoprotection and ectoine catabolism. J. Bacteriol. 187: 1293–1304.

Jensen, R.A. (1976). Enzyme recruitment in evolution of new function. Annu. Rev. Microbiol. 30: 409–425.

Jorge, C.D., Borges, N., Bagyan, I., Bilstein, A., and Santos, H. (2016). Potential applications of stress solutes from extremophiles in protein folding diseases and healthcare. Extremophiles 20: 251–259.

Kapfhammer, D., Karatan, E., Pflughoeft, K.J., and Watnick, P.I. (2005). Role for glycine betaine transport in *Vibrio cholerae* osmoadaptation and biofilm formation within microbial communities. Appl. Environ. Microbiol. 71: 3840–3847.

Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high osmolality environments. Arch. Microbiol. 170: 319–330.

Klähn, S., and Hagemann, M. (2011). Compatible solute biosynthesis in cyanobacteria. Env. Microbiol. 13: 551–562.

Kol, S., Merlo, M.E., Scheltema, R.A., de Vries, M., Vonk, R.J., Kikkert, N.A., Dijkhuizen, L., Breitling, R., and Takano, E. (2010). Metabolomic characterization of the salt stress response in *Streptomyces coelicolor*. Appl. Environ. Microbiol. 76: 2574–2581.

Kuhlmann, A.U., and Bremer, E. (2002). Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp. Appl. Environ. Microbiol. 68: 772–783.

Kuhlmann, A.U., Bursy, J., Gimpel, S., Hoffmann, T., and Bremer, E. (2008a). Synthesis of the compatible solute ectoine in *Virgibacillus pantothenticus* is triggered by high salinity and low growth temperature. Appl. Environ. Microbiol. 74: 4560–4563.

Kuhlmann, A.U., Hoffmann, T., Bursy, J., Jebbar, M., and Bremer, E. (2011). Ectoine and hydroxyectoine as protectants against osmotic and cold stress: uptake through the SigB-controlled betaine-choline- carnitine transporter-type carrier EctT from *Virgibacillus pantothenticus*. J. Bacteriol. 193: 4699–4708.

Kuhlmann, S.I., Terwisscha van Scheltinga, A.C., Bienert, R., Kunte, H.J., and Ziegler, C. (2008b). 1.55 A structure of the ectoine binding protein TeaA of the osmoregulated TRAP-transporter TeaABC from *Halomonas elongata*. Biochemistry 47: 9475–9485.

Kumarevel, T., Nakano, N., Ponnuraj, K., Gopinath, S.C., Sakamoto, K., Shinkai, A., Kumar, P.K., and Yokoyama, S. (2008). Crystal structure of glutamine receptor protein from *Sulfolobus tokodaii* strain 7 in complex with its effector L-glutamine: implications of effector binding in molecular association and DNA binding. Nucleic Acids Res. 36: 4808–4820.

Kunte, H.J., Lentzen, G., and Galinski, E. (2014). Industrial production of the cell protectant ectoine: protection, mechanisms, processes, and products. Cur. Biotechnol. 3: 10–25.

Kurz, M. (2008). Compatible solute influence on nucleic acids: many questions but few answers. Saline Syst. 4: 6.

Kurz, M., Burch, A.Y., Seip, B., Lindow, S.E., and Gross, H. (2010). Genome-driven investigation of compatible solute biosynthesis pathways of *Pseudomonas syringae* pv. syringae and their contribution to water stress tolerance. Appl. Environ. Microbiol. 76: 5452–5462.

Lamark, T., Styrvold, O.B., and Strom, A.R. (1992). Efflux of choline and glycine betaine from osmoregulating cells of *Escherichia coli*. FEMS Microbiol. Lett. 75: 149–154.

Landa, M., Burns, A.S., Roth, S.J., and Moran, M.A. (2017). Bacterial transcriptome remodeling during sequential co-culture with a marine dinoflagellate and diatom. ISME J. 11: 2677–2690.

Le Coq, J., Pavlovsky, A., Malik, R., Sanishvili, R., Xu, C., and Viola, R.E. (2008). Examination of the mechanism of human brain aspartoacylase through the binding of an intermediate analogue. Biochemistry 47: 3484–3492.

Lecher, J., Pittelkow, M., Zobel, S., Bursy, J., Bonig, T., Smits, S.H., Schmitt, L., and Bremer, E. (2009). The crystal structure of UehA in complex with ectoine- a comparison with other TRAP-T binding proteins. J. Mol. Biol. 389: 58–73.

Leon, M.J., Hoffmann, T., Sanchez-Porro, C., Heider, J., Ventosa, A., and Bremer, E. (2018). Compatible solute synthesis and import by the moderate halophile *Spiribacter salinus*: physiology and genomics. Front. Microbiol. 9: 108.

Letunic, I., and Bork, P. (2016) Intreactive tree of life (iTOL) v3. An online tool for the display and annotation of phylogenetic and other tress. Nucleic Acids Res. 44: W242–W245.

Lippert, K., and Galinski, E.A. (1992). Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl. Microb. Biotechnol. 37: 61–65.

Lo, C.C., Bonner, C.A., Xie, G., D'Souza, M., and Jensen, R.A. (2009). Cohesion group approach for evolutionary analysis of aspartokinase, an enzyme that feeds a branched network of many biochemical pathways. Microbiol. Mol. Biol. Rev. 73: 594–651.

Louis, P., and Galinski, E.A. (1997). Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiology 143: 1141–1149.

Ma, Y., Wang, Q., Xu, W., Liu, X., Gao, X., and Zhang, Y. (2017). Stationary phase-dependent accumulation of ectoine is an efficient adaptation strategy in *Vibrio anguillarum* against cold stress. Microbiol. Res. 205: 8–18.

Mais, C. -N., Hermann, L., Altegoer, F., Seubert, A., Richter, A.A., Wernersbach, I., Czech, L., Bremer, E., and Bange, G. (2020). Degradation of the microbial stress protectants and chemical chaperones ectoine and hydroxyectoine by a bacterial hydrolase-deacetylase complex. J. Biol. Chem. 295: 9087–9104.

Malin, G., and Lapidot, A. (1996). Induction of synthesis of tetrahydropyrimidine derivatives in *Streptomyces* strains and their effect on *Escherichia coli* in response to osmotic and heat stress. J. Bacteriol. 178: 385–395.

Manzanera, M., Vilchez, S., and Tunnacliffe, A. (2004). High survival and stability rates of *Escherichia coli* dried in hydroxyectoine. FEMS Microbiol. Lett. 233: 347–352.

Maskow, T., and Babel, W. (2001). Calorimetrically obtained information about the efficiency of ectoine synthesis from glucose in *Halomonas elongata*. Biochim. Biophys. Acta 1527: 4–10.

Moran, M.A., Buchan, A., Gonzalez, J.M., Heidelberg, J.F., Whitman, W.B., Kiene, R.P., Henriksen, J.R., King, G.M., Belas, R., Fuqua, C., et al. (2004). Genome sequence of *Silicibacter pomeroyi* reveals adaptations to the marine environment. Nature 432: 910–913.

Moritz, K.D., Amendt, B., Witt, E.M.H.J., and Galinski, E.A. (2015). The hydroxyectoine gene cluster of the non-halophilic acidophile *Acidiphilium cryptum*. Extremophiles 19: 87–99.

Mosier, A.C., Justice, N.B., Bowen, B.P., Baran, R., Thomas, B.C., Northen, T.R., and Banfield, J.F. (2013). Metabolites associated with adaptation of microorganisms to an acidophilic, metal-rich environment identified by stable-isotope-enabled metabolomics. mBio 4: e00484-12.

Mustakhimov, I.I, Reshetnikov, A.S., Glukhov, A.S., Khmelenina, V.N., Kalyuzhnaya, M.G., and Trotsenko, Y.A. (2010). Identification and characterization of EctR1, a new transcriptional regulator of the ectoine biosynthesis genes in the halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z. J. Bacteriol. 192: 410–417.

Müller, V., Spanheimer, R., and Santos, H. (2005) Stress response by solute accumulation in archaea. Curr. Opin. Microbiol. 8: 729–736.

Ono, H., Sawada, K., Khunajakr, N., Tao, T., Yamamoto, M., Hiramoto, M., Shinmyo, A., Takano, M., and Murooka, Y. (1999). Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately halophilic eubacterium, *Halomonas elongata*. J. Bacteriol. 181: 91–99.

Oren, A. (1999). Bioenergetic aspects of halophilism. Microbiol. Mol. Biol. Rev. 63: 334–348.

Pastor, J.M., Salvador, M., Argandona, M., Bernal, V., Reina-Bueno, M., Csonka, L.N., Iborra, J.L., Vargas, C., Nieto, J.J., and Canovas, M. (2010). Ectoines in cell stress protection: uses and biotechnological production. Biotechnol. Adv. 28: 782–801.

Peters, P., Galinski, E.A., and Trüper, H.G. (1990). The biosyntheis of ectoine. FEMS Microbiol. Lett. 71: 157–162.

Prabhu, J., Schauwecker, F., Grammel, N., Keller, U., and Bernhard, M. (2004). Functional expression of the ectoine hydroxylase gene (*thpD*) from *Streptomyces chrysomallus* in *Halomonas elongata*. Appl. Environ. Microbiol. 70: 3130–3132.

Proshlyakov, D.A., McCracken, J., and Hausinger, R.P. (2017). Spectroscopic analyses of 2-oxoglutarate-dependent oxygenases: TauD as a case study. J. Biol. Inorg. Chem. 22: 367–379.

Rawlings, N.D., and Barrett, A.J. (1993). Evolutionary families of peptidases. Biochem. J. 290: 205–218.

Record, M.T. , Jr., Courtenay, E.S., Cayley, S., and Guttman, H.J. (1998). Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments. Trends Biochem. Sci. 23: 190–194.

Ren, M., Feng, X., Huang, Y., Wang, H., Hu, Z., Clingenpeel, S., Swan, B.K., Fonseca, M.M., Posada, D., Stepanauskas, R., et al. (2019). Phylogenomics suggests oxygen availability as a driving force in Thaumarchaeota evolution. ISME J. 13: 2150–2161.

Reshetnikov, A.S., Khmelenina, V.N., Mustakhimov, , II, Kalyuzhnaya, M., Lidstrom, M., and Trotsenko, Y.A. (2011a). Diversity and phylogeny of the ectoine biosynthesis genes in aerobic, moderately halophilic methylotrophic bacteria. Extremophiles 15: 653–663.

Reshetnikov, A.S., Khmelenina, V.N., Mustakhimov, I.I., and Trotsenko, Y.A. (2011b). Genes and enzymes of ectoine biosynthesis in halotolerant methanotrophs. Methods Enzymol. 495: 15–30.

Reshetnikov, A.S., Khmelenina, V.N., and Trotsenko, Y.A. (2006). Characterization of the ectoine biosynthesis genes of haloalkalotolerant obligate methanotroph *"Methylomicrobium alcaliphilum* 20Z*"*. Arch. Microbiol. 184: 286–297.

Reshetnikov, A.S., Rozova, O.N., Trotsenko, Y.A., But, S.Y., Khmelenina, V.N., and Mustakhimov, I.I. (2020). Ectoine degradation pathway in halotolerant methylotrophs. PloS One 15: e0232244.

Reuter, K., Pittelkow, M., Bursy, J., Heine, A., Craan, T., and Bremer, E. (2010). Synthesis of 5-hydroxyectoine from ectoine: crystal structure of the non-heme iron(II) and 2-oxoglutarate-dependent dioxygenase EctD. PloS One 5: e10647.

Richter, A.A., Kobus, S., Czech, L., Hoeppner, A., Zarzycki, J., Erb, T.J., Lauterbach, L., Dickschat, J.S., Bremer, E., and Smits, S.H.J. (2020). The architecture of the diaminobutyrate acetyltransferase active site provides mechanistic insight into the biosynthesis of the chemical chaperone ectoine. J. Biol. Chem. 295: 2822–2838.

Richter, A.A., Mais, C.-N., Czech, L., Geyer, K., Hoeppner, A., Smits, A.H.J., Erb, T.J., and Bremer, E. (2019). Biosynthesis of the stress-protectant and chemical chaperone ectoine: biochemistry of the transaminase EctB. Front. Microbiol. 10: 2811.

Robin, A.Y., Cobessi, D., Curien, G., Robert-Genthon, M., Ferrer, J.L., and Dumas, R. (2010). A new mode of dimerization of allosteric enzymes with ACT domains revealed by the crystal structure of the aspartate kinase from *Cyanobacteria*. J. Mol. Biol. 399: 283–293.

Rodriguez-Moya, J., Argandona, M., Reina-Bueno, M., Nieto, J.J., Iglesias-Guerra, F., Jebbar, M., and Vargas, C. (2010). Involvement of EupR, a response regulator of the NarL/FixJ family, in the control of the uptake of the compatible solutes ectoines by the halophilic bacterium *Chromohalobacter salexigens*. BMC Microbiol. 10: 256.

Roeßler, M., and Müller, V. (2001). Osmoadaptation in bacteria and archaea: common principles and differences. Env. Microbiol. 3: 743–754.

Rubinstein, S.M., Kolodkin-Gal, I., McLoon, A., Chai, L., Kolter, R., Losick, R., and Weitz, D.A. (2012). Osmotic pressure can regulate matrix gene expression in *Bacillus subtilis*. Mol. Microbiol. 86: 426–436.

Sahle, C.J., Schroer, M.A., Jeffries, C.M., and Niskanen, J. (2018). Hydration in aqueous solutions of ectoine and hydroxyectoine. Phys. Chem. Chem. Phys. 20: 27917–27923.

Salah Ud-Din, A.I., Tikhomirova, A., and Roujeinikova, A. (2016). Structure and functional diversity of GCN5-related N-acetyltransferases (GNAT). Int. J. Mol. Sci. 17: 1018.

Saum, S.H., and Müller, V. (2008). Growth phase-dependent switch in osmolyte strategy in a moderate halophile: ectoine is a minor osmolyte but major stationary phase solute in *Halobacillus halophilus*. Env. Microbiol. 10: 716–726.

Schada von Borzyskowski, L., Severi, F., Kruger, K., Hermann, L., Gilardet, A., Sippel, F., Pommerenke, B., Claus, P., Cortina, N.S., Glatter, T., et al. (2019). Marine Proteobacteria metabolize glycolate via the beta-hydroxyaspartate cycle. Nature 575: 500–504.

Schiraldi, C., Maresca, C., Catapano, A., Galinski, E.A., and De Rosa, M. (2006). High-yield cultivation of *Marinococcus* M52 for production and recovery of hydroxyectoine. Res. Microbiol. 157: 693–699.

Schuh, W., Puff, H., Galinski, E.A., and Trüper, H.G. (1985). Die Kristallstruktur des Ectoin, einer neuen osmoregulatorisch wirksamen Aminosäure. Z. Naturforsch. 40c: 780–784.

Schulz, A., Hermann, L., Freibert, S.-A., Bönig, T., Hoffmann, T., Riclea, R., Dickschat, J.S., Heider, J., and Bremer, E. (2017a). Transcriptional regulation of ectoine catabolism in response to multiple metabolic and environmental cues. Env. Microbiol. 19: 4599–4619.

Schulz, A., Stöveken, N., Binzen, I.M., Hoffmann, T., Heider, J., and Bremer, E. (2017b). Feeding on compatible solutes: a substrate-induced pathway for uptake and catabolism of ectoines and its genetic control by EnuR. Environ. Microbiol. 19: 926–946.

Schwibbert, K., Marin-Sanguino, A., Bagyan, I., Heidrich, G., Lentzen, G., Seitz, H., Rampp, M., Schuster, S.C., Klenk, H.P., Pfeiffer, F., et al. (2011). A blueprint of ectoine metabolism from the genome of the industrial producer *Halomonas elongata* DSM 2581 T. Env. Microbiol. 13: 1973–1994.

Seip, B., Galinski, E.A., and Kurz, M. (2011). Natural and engineered hydroxyectoine production based on the *Pseudomonas stutzeri ectABCD-ask* gene cluster. Appl. Environ. Microbiol. 77: 1368–1374.

Seminara, A., Angelini, T.E., Wilking, J.N., Vlamakis, H., Ebrahim, S., Kolter, R., Weitz, D.A., and Brenner, M.P. (2012). Osmotic spreading of *Bacillus subtilis* biofilms driven by an extracellular matrix. Proc. Natl. Acad. Sci. U.S.A. 109: 1116–1121.

Shao, Z., Deng, W., Li, S., He, J., Ren, S., Huang, W., Lu, Y., Zhao, G., Cai, Z., and Wang, J. (2015). GlnR-mediated regulation of *ectABCD* transcription expands the role of the GlnR regulon to osmotic stress management. J. Bacteriol. 197: 3041–3307.

Shikuma, N.J., Davis, K.R., Fong, J.N.C., and Yildiz, F.H. (2013). The transcriptional regulator, CosR, controls compatible solute biosynthesis and transport, motility and biofilm formation in *Vibrio cholerae*. Env. Microbiol. 15: 1387–1399.

Shrestha, M., Compton, K.K., Mancl, J.M., Webb, B.A., Brown, A.M., Scharf, B.E., and Schubot, F.D. (2018). Structure of the sensory domain of McpX from *Sinorhizobium meliloti*, the first known bacterial chemotactic sensor for quaternary ammonium compounds. Biochem. J. 475: 3949–3962.

Shrivastava, T., and Ramachandran, R. (2007). Mechanistic insights from the crystal structures of a feast/famine regulatory protein from *Mycobacterium tuberculosis* H37Rv. Nucleic Acids Res. 35: 7324–7335.

Sims, D., Brettin, T., Detter, J.C., Han, C., Lapidus, A., Copeland, A., Glavina Del Rio, T., Nolan, M., Chen, F., Lucas, S., et al. (2009). Complete genome sequence of *Kytococcus sedentarius* type strain (541). Stand. Genomic Sci. 1: 12–20.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., LI, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alingmnets using Clustal Omega. Mol. Syst. Biol. 7: 539.

Smiatek, J., Harishchandra, R.K., Rubner, O., Galla, H.J., and Heuer, A. (2012). Properties of compatible solutes in aqueous solution. Biophys. Chem. 160: 62–68.

Stadmiller, S.S., Gorensek-Benitez, A.H., Guseman, A.J., and Pielak, G.J. (2017). Osmotic shock induced protein destabilization in living cells and its reversal by glycine betaine. J. Mol. Biol. 429: 1155–1161.

Steffen-Munsberg, F., Vickers, C., Kohls, H., Land, H., Mallin, H., Nobili, A., Skalden, L., van den Bergh, T., Joosten, H.J., Berglund, P., et al. (2015). Bioinformatic analysis of a PLP-dependent enzyme superfamily suitable for biocatalytic applications. Biotechnol. Advances 33: 566–604.

Stöveken, N., Pittelkow, M., Sinner, T., Jensen, R.A., Heider, J., and Bremer, E. (2011). A specialized aspartokinase enhances the biosynthesis of the osmoprotectants ectoine and hydroxyectoine in *Pseudomonas stutzeri* A1501. J. Bacteriol. 193: 4456–4468.

Street, T.O., Bolen, D.W., and Rose, G.D. (2006). A molecular mechanism for osmolyte-induced protein stability. Proc. Natl. Acad. Sci. U.S.A. 103: 13997–14002.

Tanne, C., Golovina, E.A., Hoekstra, F.A., Meffert, A., and Galinski, E.A. (2014). Glass-forming property of hydroxyectoine is the cause of its superior function as a dessication protectant. Front. Microbiol. 5: 150.

Tao, P., Li, H., Yu, Y., Gu, J., and Liu, Y. (2016). Ectoine and 5-hydroxyectoine accumulation in the halophile *Virgibacillus halodenitrificans* PDB-F2 in response to salt stress. Appl. Microbiol. Biotechnol. 100: 6779–6789.

Tramonti, A., Nardella, C., di Salvo, M.L., Pascarella, S., and Contestabile, R. (2018). The MocR-like transcription factors: pyridoxal 5'-phosphate-dependent regulators of bacterial metabolism. FEBS J. 285, 3925–3944.

Treangen, T.J., and Rocha, E.P.C. (2011). Horizontal transfer, not duplication, drives the expansion of protein families in prokaryotes. PLoS Genet. 7: e1001284.

van den Berg, J., Boersma, A.J., and Poolman, B. (2017). Microorganisms maintain crowding homeostasis. Nat. Rev. Microbiol. 15: 309–318.

Van Goethem, M.W., Swenson, T.L., Trubl, G., Roux, S., and Northen, T.R. (2019). Characteristics of wetting-induced bacteriophage blooms in biological soil crust. mBio 10: e02287-19.

Vandrich, J., Pfeiffer, F., Alfaro-Espinoza, G., and Kunte, H.J. (2020). Contribution of mechanosensitive channels to osmoadaptation and ectoine excretion in *Halomonas elongata*. Extremophiles 24: 421–432.

Vargas, C., Jebbar, M., Carrasco, R., Blanco, C., Calderon, M.I., Iglesias-Guerra, F., and Nieto, J.J. (2006). Ectoines as compatible solutes and carbon and energy sources for the halophilic bacterium *Chromohalobacter salexigens*. J. Appl. Microbiol. 100: 98–107.

Vetting, M.W., LP, S.d.C., Yu, M., Hegde, S.S., Magnet, S., Roderick, S.L., and Blanchard, J.S. (2005). Structure and functions of the GNAT superfamily of acetyltransferases. Arch. Biochem. Biophys. 433: 212–226.

Vyas, R., Tewari, R., Weiss, M.S., and Karthikeyan, S. (2012). Structures of ternary complexes of aspartate-semialdehyde dehydrogenase (Rv3708c) from *Mycobacterium tuberculosis* H37Rv. Acta Crystallogr. D Biol. Crystallogr. 68: 671–679.

Wang, B., Qin, W., Ren, Y., Zhou, X., Jung, M.-Y., Han, P., Eloe-Fadrosh, E.A., Li, M., Zhen, Y., Lu, L., et al. (2019) Expansion of *Thaumarchaeota* habitat range is correlated with horizontal transfer of ATPase operons. ISME J. 13: 3057–3079.

Warren, C. (2019). Isotope pool dilution reveals rapid turnover of small quaternary ammonium compounds. Soil Biol. Biochem. 131: 90–99.

Webb, B.A., Karl Compton, K., Castaneda Saldana, R., Arapov, T.D., Keith Ray, W., Helm, R.F., and Scharf, B.E. (2017). *Sinorhizobium meliloti* chemotaxis to quaternary ammonium compounds is mediated by the chemoreceptor McpX. Mol. Microbiol. 103: 333–346.

Weinisch, L., Kirchner, I., Grimm, M., Kuhner, S., Pierik, A.J., Rossello-Mora, R., and Filker, S. (2019). Glycine betaine and ectoine are the major compatible solutes used by four different halophilic heterotrophic ciliates. Microb. Ecol. 77: 317–331.

Welsh, D.T. (2000). Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol. Rev. 24: 263–290.

Widderich, N., Czech, L., Elling, F.J., Könneke, M., Stöveken, N., Pittelkow, M., Riclea, R., Dickschat, J.S., Heider, J., and Bremer, E. (2016a). Strangers in the archaeal world: osmostress-responsive biosynthesis of ectoine and hydroxyectoine by the marine thaumarchaeon *Nitrosopumilus maritimus*. Env. Microbiol. 18: 1227–1248.

Widderich, N., Höppner, A., Pittelkow, M., Heider, J., Smits, S.H., and Bremer, E. (2014a). Biochemical properties of ectoine hydroxylases from extremophiles and their wider taxonomic distribution among microorganisms. PloS One 9: e93809.

Widderich, N., Kobus, S., Höppner, A., Ricela, R., Seubert, A., Dickschat, J.S., Heider, J., Smits, S.H.J., and Bremer, E. (2016b). Biochemistry and crystal structure of the ectoine synthase: a metal-containing member of the cupin superfamily. PloS One 11: e0151285.

Widderich, N., Pittelkow, M., Höppner, A., Mulnaes, D., Buckel, W., Gohlke, H., Smits, S.H., and Bremer, E. (2014b). Molecular dynamics simulations and structure-guided mutagenesis provide insight into the architecture of the catalytic core of the ectoine hydroxylase. J. Mol. Biol. 426: 586–600.

Witt, E.M., Davies, N.W., and Galinski, E.A. (2011). Unexpected property of ectoine synthase and its application for synthesis of the engineered compatible solute ADPC. Appl. Microbiol. Biotechnol. 91: 113–122.

Wood, J.M. (2011). Bacterial osmoregulation: a paradigm for the study of cellular homeostasis. Annu. Rev. Microbiol. 65: 215–238.

Wu, R., Sanishvili, R., Belitsky, B.R., Juncosa, J.I., Le, H.V., Lehrer, H.J., Farley, M., Silverman, R.B., Petsko, G.A., Ringe, D., et al. (2017). PLP and GABA trigger GabR-mediated transcription regulation in *Bacillus subtilis* via external aldimine formation. Proc. Natl. Acad. Sci. U.S.A. 114: 3891–3896.

Yan, J., Nadell, C.D., Stone, H.A., Wingreen, N.S., and Bassler, B.L. (2017). Extracellular-matrix-mediated osmotic pressure drives *Vibrio cholerae* biofilm expansion and cheater exclusion. Nat. Commun. 8: 327.

Yancey, P.H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J. Exp. Biol. 208: 2819–2830.

Yokoyama, K., Ishijima, S.A., Clowney, L., Koike, H., Aramaki, H., Tanaka, C., Makino, K., and Suzuki, M. (2006). Feast/famine regulatory proteins (FFRPs): *Escherichia coli* Lrp, AsnC and related archaeal transcription factors. FEMS Microbiol. Rev. 30: 89–108.

Yu, Q., Cai, H., Zhang, Y., He, Y., Chen, L., Merritt, J., Zhang, S., and Dong, Z. (2017). Negative regulation of ectoine uptake and catabolism in *Sinorhizobium meliloti*: characterization of the EhuR gene. J. Bacteriol. 199: e00119-16.

Zaccai, G., Bagyan, I., Combet, J., Cuello, G.J., Deme, B., Fichou, Y., Gallat, F.X., Galvan Josa, V.M., von Gronau, S., Haertlein, M., et al. (2016). Neutrons describe ectoine effects on water H-bonding and hydration around a soluble protein and a cell membrane. Sci. Rep. 6: 31434.

Zhao, L., Vecchi, G., Vendruscolo, M., Korner, R., Hayer-Hartl, M., and Hartl, F.U. (2019). The Hsp70 chaperone system stabilizes a thermosensitive subproteome in *E. coli*. Cell Rep. 28: 1335–45. e1336.

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