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 phylogenic 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.

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).

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 Giersch 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 watersolubility 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).

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).
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 Mül 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 polyols (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 [(4S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] and its derivative 5-hydroxyectoine [(4S,5S)-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-hydroxyectoine 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; Prabhü 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 constraints (Supplementary Figure S1).

**Phylogenomic distribution of ectoine-producing microorganisms in Archaea**

It is well-known that many halophilic Archaea primarily use the salt-in osmotic stress 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 Thaumarchaeon *Nitrospumilus 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 constraints 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, ecd 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-archaeota, 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 Halomomas 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...
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).
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
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 IC₅₀ 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. 2011). The abbreviations used are: CoA: Coenzyme A; DABA: diaminobutyric acid; N-γ-ADABA: N-γ-acetyl-diaminobutyric acid; PLP: pyridoxal-5'-phosphate (PLP)-dependent transaminases (also referred to in the literature as aminotransferases) (Steffen-Münsberg 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 *Methylocrobium 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 activity.
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-diaminobutyrate 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 [PIEctA::CoA and PIEctB::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 PIEctA::DABA and PIEctA::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-diaminobutyrate 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 juxtaposed 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-diaminobutyrate 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 in an 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 osmstresstolerance. 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 osmstresstolerance 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 CO₂ 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 catalyst, 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 phytanoyl-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-oxodic 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, (4S,5S)-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 have found ways to re-use these compounds when they are no longer needed 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 8,557 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 (a-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).
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 Rhodobacterales (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 (Vargas et al. 2006), R. pomeroyi (Schulz et al. 2017b) and M. alcaliphilum (Reshetnikov et al. 2020). 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* (HeEutD) 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 ectoine 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...
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 abbreviations used are: N-α-acetyl-L-2,4-diaminobutyric acid (N-α-ADABA); L-2,4-diaminobutyric acid (DABA).

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 | 6Y09 | 2.4 Å      | H. elongata | (Mais et al. 2020) |
| EutE (3.5.1.125)   | apo    | 6TML           | 2.0 Å      | R. pomeroyi | (Mais et al. 2020) |
|                    | DABA, acetate | 6TWM | 2.5 Å      | R. pomeroyi | (Mais et al. 2020) |

The N-acetylated-L-2,4-diaminobutyrate deacetylase: EutE

The N-acetylated-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 crystallized 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 fixing 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 RpEutD 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 *RpEutD* 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/hydantoin 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-hydroxy-aspartate pathway of microorganisms (Schada von Borzychowski 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 route 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 *Streptomyces* 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 5-N-α-ADABA and DABA as systems-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 (Edayathamangalam 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 (Edayathamangalam 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 5-N-α-ADABA and DABA as system-specific inducers and ligands for EhuR/EnuR (Schulz et al. 2017a) results in the binding of 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 5-N-α-ADABA, 5-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 (Gieselmann 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/decactylase 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-hydroxyectoine 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; Schibbert 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, osmoprotection 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; Schibbert 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. LHI28 (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 osmoprotectants, or are there mechanistically unifying underpinnings?

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