Hydroxyectoine Metabolism in *Halomonas elongata*

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“Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand.”

Albert Einstein.


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2 Abstract

The marine Gram-negative bacterium *Halomonas elongata* is known to produce hydroxyectoine (8), a molecule that is valuable because of its broad spectrum of potential industrial applications. However, the widespread use of hydroxyectoine has been partially limited because of the high cost of production. This is in part due to the purification steps required to remove its precursor ectoine (6), which even under the best conditions for hydroxyectoine production, is still present in a relatively high proportion (>20 %). Since conversion of hydroxyectoine to ectoine has been observed, a better understanding of the mechanisms used by *H. elongata* to regulate the intracellular concentration of hydroxyectoine might provide a means to optimize its production. With this goal in mind, enzymes potentially involved in the catabolism of hydroxyectoine were identified and integrated with enzymes catalyzing the known steps in the metabolism of ectoines, creating a model that helps to understand the currently undefined pathways for hydroxyectoine degradation and its conversion to ectoine.
The genes *doeA*, *eutB* and *eutC* from *Halomonas elongata* were studied *in vivo* as recombinant proteins expressed in *Escherichia coli*, but only DoeA was proven to act on the hydroxyectoine structure, leading to its cleavage. The precise cleavage product could not be defined because at least three metabolites were produced as a result of the DoeA activity. In similar conditions, it could be proved that the *E. coli* strain expressing DoeA cleaved ectoine accumulating Nγ-acetyl-2,4-diaminobutyric acid (5), Nα-acetyl-2,4-diaminobutyric acid (7) and 2,4-diaminobutyric acid (4). Therefore, it is likely that the hydroxyectoine cleavage products include Nγ-acetyl-2,4-diamino-3-hydroxybutyric acid (9), Nα-acetyl-2,4-diamino-3-hydroxybutyric acid (10) and 2,4-diamino-3-hydroxybutyric acid (11). Overall, the ectoines’ cleavage products due to DoeA activity must include at least one molecule that is further modified by enzymes coded by the genome of *E. coli*.

The genome of *H. elongata* was modified so as to create seven deletion mutants that were defective in genes potentially involved in the metabolism of both ectoine and hydroxyectoine. The mutants (ΔeutC, ΔdoeAΔectC, ΔdoeAΔeutC, ΔeutBΔectC, ΔeutBΔectCΔdoeA, ΔeutBΔdoeA and ΔectAΔdoeA) became part of a group of 16 strains (including also *H. elongata* WT, ΔdoeA, ΔdoeB, ΔeutB, ΔectC, ΔeutBΔeutC::ΩΔdoeD, ΔectA, ΔectB, ΔectR) that was used to study the metabolism of ectoines in *H. elongata*. The analysis of their ability to use hydroxyectoine as a carbon source suggests that, in addition to genes already known to be involved in the catabolism of ectoine, the genes *eutB* and *eutC* play also a role in the degradation of hydroxyectoine. Although the precise role of these two genes remains unclear, their function in the utilization of hydroxyectoine is strongly supported by the fact that some mutants accumulate a novel metabolite (2,4-diamino-3-hydroxybutyric acid, 11), which reaches very high levels in the strain with inactivated *ectB*, *eutB*, *eutC* and *doeD* when challenged to grow on hydroxyectoine as carbon source.
3 Introduction

3.1 Osmoadaptation

The role of inorganic compounds in the life-cycle of organisms may be of profound importance for understanding their physiology (Brown, 1976). The distribution of inorganic substances across the biosphere can differ greatly, and so habitats can be significantly different in their physicochemical characteristics (Berger et al., 1997; Saum and Müller, 2008). Good examples of these abiotic substances include the salts, and among these one of the most common is sodium chloride, NaCl. The abundance of salts in a particular habitat is largely dependent on temperature and on the availability and flow of water, which in turn is strongly affected by the climate (Witherow and Lyons, 2011). Hence, rainfall and flooding in a particular habitat (e.g., in salt marshes and saline soils) cause salt dilution. Conversely, in tropical marshes, where the temperature of the water may reach well above 40 °C, accumulated sea water (e.g. in rocky tide pools) may evaporate during low tide, resulting in habitats with a higher concentration of salts (Saum and Müller, 2007). As comparable natural events have been occurring repeatedly over millions of years ago, it is not surprising that organisms have evolved complex systems of variable cytoplasm composition, protein structure, membrane composition, and gene regulation patterns (Wohlfarth et al., 1990; Cayley and Record, 2004; Yousif et al., 2010). As a result of these adaptations, abiotic substances are managed such that their intracellular concentrations allow cells to function optimally.

Most microorganisms can take up nutrients and other substances only if they are in solution (Brown, 1976). In this sense, the cell membranes play a crucial role in regulating the chemical composition of the cytoplasm (Wiggins et al., 1990; Müller and Oren, 2003; Cánovas et al., 2005). The cytoplasm membrane of most species is mainly constituted of lipids (Narita, 2011) and in halotolerant microorganisms changes in its composition have been observed as a result of osmoadaptation (Ventosa et al., 1998). Although small uncharged molecules like gases and ethanol may diffuse through this inner membrane, it is regarded as a barrier for polar substances (e.g. ions), whose chemical activity is known to differ on the inside versus the outside of the cells (Wang...
and Tajkhorshid, 2007). In order to get across the membrane, substances (especially ions and large molecules) require the mediation of proteic transport systems. These often comprise pore-like structures of variable specificity, which can selective based on pore size and electrostatic barriers (Portella et al., 2007). Despite the existence of transport systems for water (apparently missing in most species of prokaryotes: Soupene et al., 2002), diffusion of water through the membrane is still possible. This requires activation energy (Engel et al., 2000). Cell membranes may also contain charged groups, especially negatively (e.g. phosphate), which can interact strongly with divalent cations (e.g. Ca\(^{2+}\) and Mg\(^{2+}\)). However, their interactions with monovalent cations (e.g. Na\(^{+}\) and K\(^{+}\)) are considered weak (Böckman et al., 2003). The movement of dissolved solute particles in nonideal solutions like those in most of the biological systems is influenced by their size, shape, charge and other factors. Therefore, the many solutes surrounding a membrane contribute differently to the chemical potential, which directly influences the osmotic pressure. The osmotic pressure is consequently described in terms of osmolality, which is considered to affect the physiological processes that involve movement of solutes and water across the membrane (Sweeney and Beuchat, 1993).

The peculiar properties of the cell membrane (summarized above) can result in both a concentration gradient and a difference in pressure across the membrane. Concentration gradients may be used by the cell to, for example, produce energy (e.g. H\(^{+}\) gradient) or to avoid the concentration of specific solutes (e.g. Cl\(^{-}\)) from reaching toxics levels (that interfere with the growth) by regulating the activity of the corresponding transport systems. On the other hand, in order to reproduce cells need to grow bigger before cell division. Hence, a greater pressure from the cytoplasm side of the membrane than that from the medium side is required (positive turgor: Kempf and Bremer, 1998; Borges et al., 2002).

Bacteria may behave differently depending on the external salinity and several classification systems based on these phenotypes have been developed (Ventosa et al., 1998). However, sharp distinctions among classes are difficult because the minimum, optimum and maximum salt concentrations for growth also depend on factors like temperature and media composition. Moreover, some organisms have been called
halophiles only because they are from the sea (≈0.6 M ≈3.5 % w/v NaCl). The most widely used system defines as **moderate halophiles** organisms growing optimally between 0.5 and 2.5 M salt. Organisms are considered as **halotolerant** if they are able to grow both without salt and in the presence of relatively high salt concentrations (< 2.5 M NaCl); **extremely halotolerant** if they can grow at salt concentrations above 2.5 M NaCl; and as **extreme halophiles** if best growth occurs at very high salt concentrations (2.5 -5.2 M NaCl) (Oren, 2008).

Studies on osmoadaptation have demonstrated that some organisms (e.g., *Vibrio costicola* (Gilboa et al., 1991) and *Brevibacterium* sp. (Nagata et al., 1995)) growing in saline media (>1 M NaCl) can maintain a low intracellular concentration of sodium ions (<0.5 M). Furthermore, it has been confirmed that *Escherichia coli* and *Bacillus subtilis* accumulate very little Na⁺ in the cytoplasm following an osmotic upshift. Instead, the intracellular concentration of K⁺ increases, which after being transported into the cytoplasm obtained as a counterion de novo biosynthesized glutamate (Kempf and Bremer, 1998). All of these microorganisms prefer, however, to replace most of the accumulated potassium glutamate for highly soluble small organic molecules with no net charge. These have been shown not to disturb the proper functioning of macromolecules and cell structures even when accumulated at molar concentrations (compatible solutes; e.g. trehalose, ectoine and proline). This strategy of dealing with external high salinity (**organic osmolyte**) allows considerable osmotic flexibility for organisms because the accumulation of compatible solutes, either through biosynthesis or by uptake from the medium (if available), can be adjusted by several mechanisms to counterbalance the osmotic pressure within wide ranges (Nagata et al., 1995; Gramman et al., 2002; Empadinhas and da Costa, 2008), and over short time scales (Sauer and Galinski, 1998). *In-vitro* studies on enzymes from moderate halophiles (e.g. *H. elongata*) showed that there was a decrease in activity in reaction mixtures with high NaCl concentration, supporting the idea of low abundance of these ions in their cytoplasm (Gilboa et al., 1991; Ono et al., 1999). Researches concerned with food preservation have shown that in some bacteria (e.g., *E. coli* and *Salmonella enterica*) an osmotic upshift leads to a variable lag phase, even if they are able to biosynthesize compatible solutes, and that the maximum growth rate is reduced (Zhou et al., 2011).
The accumulation of potassium glutamate seems to be just a first osmo-adaptation response in halotolerant microorganisms. However, other prokaryotes, including halophilic Archea species from the genus *Halobacterium* and *Haloarcula*, opt for the accumulation of potassium chloride (KCl), and are therefore recognized as users of the “salt-in cytoplasm” strategy to thrive in high salinity environments. This latter group, which is completely or partially unable to synthesize compatible solutes, is restricted to live in environments of high salinity (>2 M NaCl). These extreme halophilic microorganisms have adapted their macromolecule structures (e.g. proteins with abundant acidic amino acids) so that they can perform optimally under corresponding ionic strengths (Gramman et al., 2002, Oren et al., 2002).

Because the macromolecules of microorganisms using the salt-in cytoplasm strategy have a dependence on a high and stable ionic strength, this osmoadaptation mechanism is believed to be less advantageous than the organic osmolyte strategy for colonizing environments of low or fluctuating salinities (Saum and Müller, 2008; Pastor et al., 2010). Naturally saline environments may be accompanied by low temperatures (e.g. five kilometers below the surface in the sea (Martin et al., 2002) or cold saline lakes in the Antarctic); high temperatures (e.g., shallow or abyssal marine geothermal areas (Empadinhas and da Costa, 2006)); or low and high pH (e.g., soda lakes (Padan et al., 2005)). Organisms thriving with the organic osmolyte strategy have been described from all of these habitats (Ventosa et al., 1998).

### 3.2 Compatible solutes and the importance of ectoines

Over twenty compatible solutes have been already characterized. These “fall into a few structural classes, such as sugars (trehalose, sucrose), polyols (glycerol, sorbitol, manitol, α-glucosylglycerol, mannosyl-glycerol, and mannosyl-glyceramide), N-acetylated diamino acids (e.g., N-acetylglutaminylglutamine amide), betaines (like glycine betaine and derivatives), amino acids (proline, glutamate, glutamine, and alanine) and derivatives. The latter group includes ectoines (ectoine and hydroxyectoine). It has been demonstrated that most bacteria use an array of different solutes for osmotic balance, mainly depending on the duration of the osmotic stress, the level of salinity, the availability of substrates and osmolytes in the surroundings or the carbon source used for the growth medium (Roberts, 2005).” (Pastor et al., 2010)
“The predominant compatible solutes in halophilic Bacteria are the amino acid derivatives glycine-betaine and ectoine (Roberts, 2006; Oren, 2008). Most chemo heterotrophic Bacteria can readily use glycine-betaine as a compatible solute if it is available in the environment. However, only a few are capable of de novo synthesis of glycine-betaine (Nyyssölä et al., 2000). Far more prokaryotes synthesize the aspartate derivative ectoine (1,4,5,6,tetra-2-methyl-4-pyrimidonecarboxylic acid) as their main compatible solute (Galinski et al., 1985; Severin et al., 1992; Roberts, 2006), which can also be utilized as an energy source by halophilic bacteria such as \textit{H. elongata} and \textit{Chromohalobacter salexigens} (Vargas et al., 2006). Compatible solutes are beneficial for bacterial cells not only as osmoregulatory solutes, but also as protectants of proteins by mitigating detrimental effects of freezing, drying and high temperatures (Lippert and Galinski, 1992; Borges et al., 2002). The beneficial effect is explained by the unfavorable interaction of compatible solutes with the protein’s peptide backbone. The lower affinity of compatible solutes, compared with water, for the protein surface is termed the osmophobic effect and results in a thermodynamic force that contributes to protein folding and increased protein stability (Bolen and Baskakov, 2001; Bolen, 2004).” (Schwibbert et al., 2011)

Ectoine was discovered in \textit{Halorhodospira} (formerly \textit{Ectothiorhodospira}) \textit{halochloris} (Galinski et al., 1985). It "possesses additional protective properties compared with other compatible solutes and stabilizes even whole cells against stresses, such as UV radiation or cytotoxins (Buommino et al., 2005; Furusho et al., 2005; Kanapathipillai et al., 2005; Kolp et al., 2006). It also protects against nanoparticle-induced inflammation in lung epithelia (Sydlik et al., 2009), and damage to the small bowel from ischaemia and reperfusion injury (Wei et al., 2009). Its protective properties make ectoine a valuable compound, which is marketed in health care and skin care products. Ectoine is therefore produced annually on a scale of tons by industry in a biotechnological process with the halophilic \(\gamma\)-proteobacterium \textit{H. elongata} used as producer strain (Vreeland et al., 1980; Lentzen and Schwarz, 2006)" (Schwibbert et al., 2011; Pastor et al., 2010)

Hydroxyectoine was discovered in \textit{Streptomyces parvulus} (Inbar and Lapidot, 1988). As most important compatible solute, hydroxyectoine is more common among Gram-positive halophilic/halotolerant bacteria, but it is also often synthesized at lower amounts together with ectoine in many other ectoine-producing species. Since it was first
observed that *C. salexigens*, *H. elongata* and *Streptomyces griseous* accumulate hydroxyectoine in response to temperature upshift, and that a *C. salexigens* mutant devoid of the main ectoine hydroxylase is thermosensitive, a biological function for hydroxyectoine as a thermoprotectant was suspected (Pastor et al., 2010, Reuter et al., 2010). This *in vivo* role of hydroxyectoine is also supported by *in vitro* studies that showed its ability to protect lactate dehydrogenase from thermal inactivation (an ability not observed with ectoine), at a level comparable to that observed with mannosylglycerate, a compatible solute found in hyperthermophiles (Borges, 2002). Hydroxyectoine biosynthesis has been also noted to increase in *Marinococcus* M52, *S. coelicolor*, and *V. salexigens* during the stationary phase of growth. Since this phase provides multiple adaptation challenges to microbial cells, additional physiological roles to that of thermoprotection can be expected (Reuter et al., 2010). Thus despite being almost chemically identical to ectoine, hydroxyectoine seems to provide additional protective properties derived from its hydroxylated nature.

Because of the properties summarized above, the ectoines have an enormous potential for biotechnological use and the understanding of their intriguing function and complex metabolism remain a very active research area.

### 3.3 *Halomonas elongata* and the metabolism of ectoines

Studies concerned with the taxonomic distribution of compatible solutes indirectly revealed the potential of *H. elongata* (*Halomonadaceae* family; Franzmann et al., 1988) as an industrial producer of ectoine (Wohlfarth et al., 1990; Severin et al., 1992). This bacterium was isolated from a salt condenser (25.6 % w/v salts, ≈4.4 M NaCl) located on Bonaire Island in the Caribbean Sea in 1974. *H. elongata* cells were subsequently isolated and described as having the following characteristics: elongated rods, facultative aerobic, Gram-negative, forming white-opaque colonies in solid media, able to grow on complex media at pH 5-9, salinity 3.5-32 % (best growth at 3.5 %), and temperature 4-45 °C (optimum 30 °C). Corresponding liquid cultures in the log phase of growth consisted predominantly of singled and paired cells with polar flagella (Vreeland et al., 1980). After the use of 16S and 23S rRNA genes for phylogenetic analysis, *H. elongata* (ATCC 33173=DSM 2581\(^T\)) remained grouped into the same genus and in the gamma subdivision of the Proteobacteria phylum (de la Haba et al., 2010).
Screening for compatible solutes in *H. elongata* cells resulted in the detection of both ectoine and hydroxyectoine, and their concentration was proportional to the salinity. Nonetheless, an increment in the cultivation temperature decreased the amount of ectoine and increased the amount of hydroxyectoine. Despite this, ectoine always remained the dominant compatible solute under the conditions of the experiments (20-40 °C and 5-20 % NaCl; Wohlfarth et al., 1990). These interesting results led researchers to focus on strategies for industrial production of ectoines, i.e. bacterial milking (Sauer and Galinski, 1998). They also sought a better understanding of their metabolism and did establish the transposon mutagenesis in *H. elongata* (Kunte and Galinski, 1995). This led to the discovery of the genes responsible for the biosynthesis of ectoines (Göller et al., 1998) followed by the identification of the corresponding uptake system (Gramman et al., 2002). Studies on the transport of potassium (TrkAI und TrkAH) concluded that after an osmotic upshock, the level of accumulated potassium prevails in the cytoplasm longer (>2 h) than it does in *E. coli* (1 h), even if ectoine has already been biosynthesized (Kraegeloh *et al.*, 2005).

Despite all of this remarkable progress in research on osmoadaptation, many questions remain. For example, how does the efflux system control ectoine excretion? What are the corresponding proteins and how they function? This situation, together with the successful commercialization of ectoine containing cosmetics (since the late 1990s), pushed forward a project for the sequencing of the *H. elongata* genome. Once completed, this facilitated the search for genes involved in ectoine degradation (Schwibbert *et al.*, 2011). Today there is a considerable amount of information about *H. elongata* available. It has become a model bacterium to study stress response and has even been employed as a host in an expression system (Witt *et al.*, 2011). The key cellular elements in the metabolism of ectoines now are shown in figure 1.
3.3.1 Biosynthesis pathway of ectoines.

The sequences of the three genes shown to be involved in the biosynthesis of ectoine (ectABC) were determined during the characterization of a *H. elongata* mutant that became salt sensitive following the interruption (by transposon insertion) of the first gene in the cluster (Göller et al., 1998). When grown on glucose medium, this ectA::Tn1732 mutant was unable to synthesize ectoine. The genetic organization was confirmed to be similar to that already determined in *Marinococcus halophilus*. Hence, it could be deduced that aspartate semialdehyde (AS) is transaminated to 2,4-diaminobutyric acid (DA) by action of EctB. In a second step, catalyzed by EctA, DA is acetylated forming Nγ-acetyl-2,4-diaminobutyric acid (γ-NADA), which experiences a dehydration mediated by EctC to produce ectoine (E). It was additionally noted that in
ectA::Tn1732 cultures supplemented with hydroxyectoine (HE), not only had the growth ability at high salinity been restored, but also that ectoine could be detected inside the cells. Therefore, the existence of a HE-E conversion pathway without the participation of EctA was proposed and supported by subsequent studies on the related bacterium C. salexigens (formerly H. elongata DSM 3043; Cánovas et al., 1999). The pathway for the biosynthesis of ectoine via AS-DA-NADA-E was confirmed by in vitro experiments with the corresponding enzymes. The reversibility of EctB could be demonstrated but no product of the reversible reaction could be detected with EctA or EctC (Ono et al., 1999). The deletion of ectA provided evidence for an accumulation of glutamine, glutamate and 5-Amino-3,4-dihydro-2H-pyrrol-2-carboxylate (ADPC). ADPC, not detected in the wild type strain, is thought to be a product of a reversible side reaction of EctC over glutamine, an alternative substrate that accumulates in the absence of γ-NADA (present at higher concentration in the cytoplasm of the ΔectC mutant), the main substrate of EctC (Witt et al., 2011), which is known as a natural product part of latex (Cánovas et al., 1999). Interestingly, the deletion of the ectB gene do not disable ectoine biosynthesis, indicating that other enzymes catalyze the AS-DA conversion and that it might also play additional roles. Next to the ectABC region there is a gene coding for a putative regulator protein (EctR) whose function remains unclear (Schilz 2005). Mapping the transcription initiation sites of the ectoine operon by RACE-PCR identified the location of two promoters, one possibly dependent on a σ70 and one dependent of an osmotically induced σ38 transcription factor (Lee and Gralla, 2004), upstream of ectA and a third one, a σ54 (often found in nitrogen regulated genes) upstream of ectC (Schwibbert et al., 2011). Nevertheless, the existence of additional promoters cannot be discarded.

**Biosynthesis of hydroxyectoine** in H. elongata was demonstrated to occur by direct hydroxylation of ectoine. The gene coding for this hydroxylase (ectD) is located separately from the ectoine cluster. The main transcription of ectD seems to be under the control of a σ5 dependent promoter. This promoter type usually responds to general stress factors like those typical of the stationary phase or to unusual pH and temperatures. A significant improvement in the hydroxyectoine ratio to approximately 75 % of the ectoine pool in H. elongata was achieved by submitting cells growing in a log phase (10 % w/v NaCl, minimal medium) to a heat shock (37-50 °C). EctD was shown
to be able to hydroxylate homoectoine, proline, ADPC, and guanidino ectoine (Meffert, 2011). Characterization of crystals of EctD from *Virgibacillus salexigens* supported previous knowledge about the enzymatic hydroxylation of ectoine, in which molecular oxygen is consumed with release of CO₂ from an α-ketoglutarate molecule in a non-heme iron containing active site (Reuter et al., 2010). The hydroxylation of ectoine has been studied in detail *in vitro* with no evidence of reversibility (Bursy et al., 2007).

### 3.3.2 Ectoines degradation

The genes for the degradation of ectoine (*doeABCD*) were located as a result of sequence homology comparisons between genes induced in *Sinorhizobium meliloti* growing in ectoine supplemented media and predicted genes in the *H. elongata* genome. Ectoine is cleaved by DoeA apparently forming both Nα-acetyl-2,4-diaminobutyric acid (α-NADA) and Nγ-acetyl-2,4-diaminobutyric acid (γ-NADA). However, only α-NADA is deacetylated by DoeB to DA. DA is then deaminated by DoeD, forming AS, which is further oxidized by DoeC, producing aspartate. Deletion of genes *doea*, *doeb* or *doec* abolishes the growth on ectoine as carbon source. The growth inhibition of *H. elongata* Δ*doec* on ectoine as carbon source raises questions about the fate of the acetate cleaved by DoeB, which should be used as a carbon source and to support growth. However, as this strain can grow on ectoine as a nitrogen source, the existence of some kind of catabolite repression was suggested for this pathway. Deletion of *doed* leads to a three-fold reduction of the maximum growth rate on ectoine as a carbon source. Interestingly, deletion of both *ectB* and *doed*, either alone or together, does not abolish either the growth on ectoine or its biosynthesis. This again supported the participation of at least one more enzyme in the AS-DA conversion and raises questions about the role(s) of these enzymes. Between *doeb* and *doec* there is a gene coding for a transcriptional regulator protein of the AsnC/Lrp family (either repressor or activator) binding a DNA sequence right upstream of the *doea* start codon. Downstream of the *doe* cluster there are two genes of predicted activity in the utilization of ectoine, as concluded from the high homology with corresponding genes in *S. meliloti*. The deletion of these two genes did not affect the degradation of ectoine and thus their functions remain unclear. Their predicted functions are of a dehydratase (*eutB*) and of a cyclodeaminase (*eutC*) (Schwibbert et al., 2011).
Introduction

More details about the chemical species involved in the biosynthesis and degradation reactions are summarized in figure 2.

Figure 2. Metabolic pathways of ectoines in *H. elongata* as presented by Schwibbert et al. (2011), extended until the oxaloacetate. The reaction 2.6.1.1 is catalyzed by aspartate aminotransferase for which there are three putative genes in the genome of *H. elongata*. LysC: aspartate kinase; Asd: β-aspartate-semialdehyde-dehydrogenase; EctB: L-2,4-diaminobutyric acid transaminase; EctA: L-2,4-diaminobutyric acid Nγ-acetyltransferase; EctC: ectoine synthase; EctD: ectoine hydroxylase; DoeA: ectoine hydrolase; DoeB: Nα-acetyl-L-2,4-diaminobutyric acid deacetylase; DoeD: L-2,4-diaminobutyric acid transaminase; DoeC: aspartate-semialdehyde dehydrogenase.

3.3.3 Membrane transport of ectoines

**Uptake of ectoines** in *H. elongata* was demonstrated to occur exclusively through a Tripartite ATP-independent Periplasmic Transporter (TRAP-T) system, consisting of three proteins involved in the ectoine transport: a periplasmic substrate binding protein (TeaA), a small transmembrane protein (TeaB) and a large transmembrane protein (TeaC). The corresponding genes are organized in an operon (*teaABCD*) with two putative promoters upstream of *teaA*. The *teaD*, however, is thought to be transcribed together with *teaBC*. Contrary to the deletion of any of the first genes, the deletion of *teaD* did not affect uptake activity or growth in high salinity media. Its predicted protein
sequence was shown to be homologous to that of the universal stress protein, UspA. TeaD is an ATP binding protein believed to function as some kind of energy sensor (Schwibbert et al., 2011). TeaA is a high-affinity ectoine binding protein ($K_d = 0.19 \mu M$) also acting on hydroxyectoine but with less affinity ($K_d = 3.8 \mu M$) (Kuhlmann et al., 2002). *H. elongata* strains with a defective TRAP-T system release ectoine to the medium and therefore the TeaABC transporter was associated not only with the transport of exogenous ectoines as a stress protection or energy sources, but also with the recovery of de novo biosynthesized ectoines leaking out of the cell through an unknown efflux system (Kunte 2006). Despite of the leakage of ectoine, cells of strains with a defective uptake system maintain normal ectoine levels. Therefore it was suggested that the Tea transport system might be involved in the regulation of the ectoine synthesis (Kuhlmann et al., 2002; Kunte 2006) and its defect results in the overexpression of ectoine (European Patent: Kunte et al., 2002). TRAP transporters are generally considered to be unidirectional due to their high specificity and their functional dependence on Na$^+$. The only well-characterized part of TRAP-T systems is the substrate binding proteins. Due to the lack of experiments with corresponding membrane proteins, their overall structure and the translocation mechanism are not well understood. The large transmembrane protein is believed to form the translocation channel. Considering the scarce information available, a hypothetical mechanism of the TRAP mediated uptake has been proposed (Mulligan et al., 2010).

After a hypoosmotic shock, cells have to adjust the chemical potential of the cytoplasmic water rapidly. The **efflux systems** employed by *H. elongata* are not currently understood. In other organisms, efflux of cytoplasmic solutes occurs through transmembrane channels that should open as a response to critically unbalanced internal pressures. Two types of responses have been postulated. The mechanosensitive channels (Msc) are gated by membrane tension and thought to be the primary biosensors for osmoregulation in bacteria. They act with a rapid and non-discriminating release of solutes upon hypoosmotic shock. The other excretion system, the volume activated channels (Vac), may release several osmolytes and has been also proposed to be responsible for anion efflux upon hypoosmotic shock (Roberts, 2005).
3.4 Heterologous expression in *E. coli*

Studies on different strains of *E. coli* have been undertaken for more than a century. Today *E. coli* is among the most well known organisms to scientists. Among the most common applications for *E. coli*, is its use as a host for heterologous expression of proteins. Heterologous expression requires genetic manipulation of both the donor and the host. In a simple way, the DNA region coding for the protein of interest is extracted from the donor and inserted in the genome of the host through a variety of genetic engineering techniques, usually using plasmids as vectors (Rai und Padh, 2001).

There are several well-known, commercially available expression systems (e.g. from Novagen) that greatly facilitate the genetic manipulation. One example is the pET System, which is recognized as one of the most powerful approaches currently available for producing recombinant proteins. Based on the T7 promoter-driven system, the pET system has been expanded to include over 23 vector types (e.g., 22b+), 11 different *E. coli* host strains (e.g., BL21) and many other companion products designed for a more efficient detection and purification of target proteins.

*E. coli* produces trehalose as a compatible solute, which confers osmoprotection in minimal media with up to 0.5 M NaCl. Its osmoadaptation response, as well as its ability to uptake many compatible solutes, including ectoines, has been thoroughly documented (Kempf and Bremer, 1998). It is thought that the outer membrane is permeable to compatible solutes, which are transported by passive diffusion. Ectoines pass the inner membrane by the single component transport system ProP. Numerous studies have shown that *E. coli* enzymes do not alter the chemical structures of ectoines. These features have allowed scientist to successfully study the enzymes of *H. elongata* by testing their activity as recombinant proteins *in vivo* as part of the cytoplasm components of *E. coli* either for biocatalysts (Meffert 2011) or just to assay their activity (Schwibbert et al., 2011). Additional studies reported in *E. coli* have included the testing of ectoines for cell preservation ability after exposure of ectoine containing cells to low or high temperatures, dehydration and other environmental stresses.
3.5 Objectives

Experiments with strains defective in the ectoine biosynthesis pathway have been shown to still synthesize ectoine starting from hydroxyectoine provided in the media. This implies the existence of at least one mechanism to remove the hydroxyl group from hydroxyectoine. However, the mechanism(s) of this biotransformation is completely unknown. How many enzymatic steps are involved? What are the corresponding enzymes? Is there just one or more conversion pathways? What are the conditions that trigger this conversion? Is the conversion of hydroxyectoine to ectoine an essential first step in its catabolism? Is it reversible, and why or why not? These are only some of the questions that need to be answered for a better understanding of the metabolism of hydroxyectoine. Answers to these questions might be the key for a more complete picture of the role that compatible solutes play in *H. elongata*. To help better understand the conversion of hydroxyectoine to ectoine, this work seeks:

1. To identify genes potentially involved in the conversion of hydroxyectoine to ectoine in *H. elongata* DSM 2581T.

2. To express in *E. coli* the genes that are potentially involved in the hydroxyectoine-ectoine conversion observed in *H. elongata*, and to analyze the corresponding protein(s) *in vivo* for their predicted activity.

3. To construct *H. elongata* mutants that are defective in the genes above and develop methods to quantify their conversion ability.

4. To phenotype *H. elongata* mutants, aiming at the identification of patterns at any level helpful in the elucidation of the conversion or degradation mechanisms of hydroxyectoine.

5. To analyze *H. elongata* mutants, aiming to identify intermediates in the pathway for the conversion of hydroxyectoine to ectoine.
4 Material and Methods

Important general information about basic techniques in molecular cloning (e.g. buffer, reagent and media preparation) as well as safety advice can be found in Sambrook and Russell (2006).

4.1 General cultivation conditions and biological material

4.1.1 Media

All of the following media were prepared in de-mineralized water. Their salt concentration can be different. A specific NaCl concentration in a given medium is indicated as a percentage number (w/v) after a dash following the medium name abbreviation. For example, MM63-6 refers to a mineral salt medium with 6 g of NaCl in 100 mL of culture. For certain experiments, the glucose concentration was reduced, or ectoine and hydroxyectoine were provided as sources of carbon, as specified in the corresponding description of the experiments. In all these cases, however, the concentration of the other media components remained constant.

2x YT Medium. mass of components in 1L

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>16.0000</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10.0000</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0000</td>
</tr>
</tbody>
</table>

pH 7.2 (KOH / HCl)

Antibiotic Broth (AB) Medium. mass of components in 1L

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic Broth Medium No.3</td>
<td>17.5000</td>
</tr>
<tr>
<td>NaCl*</td>
<td>variable</td>
</tr>
</tbody>
</table>

*When adjusting the NaCl concentration, it is necessary to consider that 17.5 g of AB medium include 3.5 g of NaCl.

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**Bp Medium (composition provided by Bitop AG). mass of components in 1L**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose monohydrate</td>
<td>5.0000</td>
</tr>
<tr>
<td>NaCl</td>
<td>Variable</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>13.600</td>
</tr>
<tr>
<td>NaOH</td>
<td>3.0000</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2.7000</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.8000</td>
</tr>
<tr>
<td>Dihydrated tri-sodium-citrate</td>
<td>0.1600</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.0610</td>
</tr>
</tbody>
</table>

Components 1-8 are dissolved, pH is adjusted to 7.0 and sterile filtered.

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄·7H₂O: 100 x solution (0.8 g/10 mL) is sterilized by filtration</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

Component 9 is stored (RT) and added right before inoculation.

**Luria Bertani (LB) Medium. mass of components in 1L**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0000 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0000 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>variable</td>
</tr>
</tbody>
</table>

→ pH 7.2 (KOH / HCl)

**Luria Bertani Glucose (LBG) Medium. mass of components in 1L**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0000 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0000 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>variable</td>
</tr>
<tr>
<td>Glucose monohydrate</td>
<td>2.0000 g</td>
</tr>
</tbody>
</table>

→ pH 7.5 (KOH / HCl)

**Luria Bertani Glucose (LBG) sucrose Medium. mass of components in 1L**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0000 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0000 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>variable</td>
</tr>
<tr>
<td>Glucose monohydrate</td>
<td>2.0000 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>220.0000 g</td>
</tr>
</tbody>
</table>

→ pH 7.5 (KOH / HCl)
Material and Methods

Mineral Salt (MM63) Medium (Larsen et al., 1987). mass of components in 1L

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>13.6000 g</td>
</tr>
<tr>
<td>KOH</td>
<td>4.2100 g</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>1.9800 g</td>
</tr>
<tr>
<td>MgSO$_4$$\cdot$7H$_2$O</td>
<td>0.2500 g</td>
</tr>
<tr>
<td>FeSO$_4$$\cdot$7H$_2$O</td>
<td>0.0011 g</td>
</tr>
</tbody>
</table>

$\rightarrow$ pH 7.1 (KOH / HCl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>variable</td>
</tr>
</tbody>
</table>

$\rightarrow$ Autoclave without glucose

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monohydrated Glucose *</td>
<td>5.0000 g</td>
</tr>
</tbody>
</table>

*28 mL of a sterile (by autoclaving) 1 M Glucose solution

4.1.1.1 Components of modified media

**Agar.** Solid media contained agar (1.5 % w/v). Plates with solid media were stored at low temperatures (4 °C) until their use.

**Antibiotics.** Stock solutions (50 mg/mL) were prepared in demineralized water and sterilized by filtration. Solutions were stored frozen (-20 °C). The appropriate volume for the desired antibiotic concentration in a given media was added to agar media before solidification (50-65°C) or to liquid cultures right before inoculation (at RT). Antibiotic concentration is indicated by an abbreviation of the antibiotic name followed by a number that indicates its concentration in µg/mL, for example: kanamycin 50 µg/mL: kan50; ampicillin 100 µg/mL: amp100.

**Ectoines.** Stock solutions (1 M) were prepared in demineralized water and sterilized by filtration. Sterile stock solutions were stored frozen (-20 °C).

4.2 Bacterial strains and their preservation

Bacterial strains used in this study (Table 1), were obtained from culture collections either at the Institute for Microbiology and Biotechnology of the University of Bonn (Bonn), or at the Biodeterioration and Reference Organisms division of the (German) Federal Institute for Materials Research and Testing (BAM, Berlin). The newly-constructed mutant strains are specific derivatives as described in the corresponding procedures (see below). Pure representatives of each strain were grown on agar plates of AB medium (with two or five percent salinity, depending on their salt tolerance, and temperatures of 30 °C for *H. elongata* and 37 °C for *E. coli*) until growth could be observed. Plates were then stored at 4 °C until further use, but for no longer than two months.
Table 1. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype and/or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. elongata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM 2581T</td>
<td>Wild type</td>
<td>Vreeland et al., 1980</td>
</tr>
<tr>
<td>KB1</td>
<td>ΔectA</td>
<td>Grammann et al., 2002</td>
</tr>
<tr>
<td>KB13</td>
<td>ΔectB</td>
<td>Schwibbert et al., 2011</td>
</tr>
<tr>
<td>WUB01</td>
<td>ΔectC</td>
<td>Witt, 2005</td>
</tr>
<tr>
<td>KB30</td>
<td>ΔectR</td>
<td>Schilz, 2005</td>
</tr>
<tr>
<td>KB43</td>
<td>ΔdoeA</td>
<td>Schwibbert et al., 2011</td>
</tr>
<tr>
<td>KB42</td>
<td>ΔdoeB</td>
<td>Schwibbert et al., 2011</td>
</tr>
<tr>
<td>KB49</td>
<td>ΔeutB</td>
<td>Schwibbert et al., 2011</td>
</tr>
<tr>
<td>K2.3</td>
<td>ΔeutB, ΔeutC, ectB::Ω, ΔdoeA</td>
<td>Schibbert, K. (BAM)</td>
</tr>
<tr>
<td>KB43.1</td>
<td>ΔdoeA, ΔectC</td>
<td>This work</td>
</tr>
<tr>
<td>KB49.1</td>
<td>ΔeutB, ΔectC</td>
<td>This work</td>
</tr>
<tr>
<td>KB49.1.1</td>
<td>ΔeutB, ΔectC, ΔdoeA</td>
<td>This work</td>
</tr>
<tr>
<td>KB49.2</td>
<td>ΔeutB, ΔdoeA</td>
<td>This work</td>
</tr>
<tr>
<td>KB1.12</td>
<td>ΔectA, ΔdoeA</td>
<td>This work</td>
</tr>
<tr>
<td>KB43.2</td>
<td>ΔdoeA, ΔeutC</td>
<td>This work</td>
</tr>
<tr>
<td>KB50</td>
<td>ΔeutC</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21</td>
<td>F-, dcm, ompT, hsdS(rB-mB-), gal/l (DE3)</td>
<td>Studier und Moffatt, 1986; Studier, 1991</td>
</tr>
<tr>
<td>DH5α</td>
<td>F-, supE44, recA1, endA1, relA1, hsdR17(ri-, mki+), gyrA96, i-, thi-1, ΔlacU169 (φ80lacZΔM15)</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>S17-1</td>
<td>RP4-2 (Tc::Mm) (Km::Tn7) Smr, pro, thi, recA</td>
<td>Simon et al., 1983</td>
</tr>
</tbody>
</table>

4.3 Generalities of biomass production and concentration of cells

Unless otherwise specified in the description of a particular experiment, cultivation in liquid media took place in thermo-shakers (Scientific innova 4230, Fa. New Brunswick [Edison, USA] or Infors AG [Bottmingen, Switzerland]) at 180 rpm and at temperatures of 30 °C (H. elongata) or 37 °C (E. coli).

Erlenmeyer flasks were used as reactors for cell growth of overnight cultures and other liquid cultures except for the expression of recombinant proteins, which took place in “OD”-flasks (nose-flasks). Overnight cultures were prepared by inoculation of media with appropriate strains stored in plates. Biomass concentration was estimated by measurement of the photon absorption (wavelength 600 nm) either using a photometer or microplate reader.
4.4 DNA cloning techniques

4.4.1 Intracellular DNA extraction and storage

4.4.1.1 Genomic DNA (Phenol-Chloroform) Extraction
Cells in an overnight culture (LB-1 medium, 4 mL) were harvested by centrifugation (Biofuge fresco, Heraeus, 10000xg, 3 min) in 2 mL capacity microfuge tubes. After discarding the supernatants, cells in pellets were subjected to a washing step by addition of TES buffer (5 mM Tris-HCl, 5 mM EDTA, 5 mM NaCl, pH 8.0; 1 mL). Suspension through vortexing was followed by cell recovery by centrifugation and then elimination of the supernatants. The washed cells were suspended again in TES buffer (800 μL), and lysozyme was added. Digestion of the peptidoglycans at the outer membrane of cells was allowed by incubation of the mixtures (RT, 45 min). SDS (20 % w/v, 20 μL) was added followed by careful mixing by vial inversion (6 times). Partial dissolution of the cytoplasmic membrane was allowed during an incubation period (RT, 30 min). A solution of phenol, chloroform and isoamyl alcohol (300 μL)\(^1\) was added and the mixtures were strongly agitated. After centrifugation (13600xg, 10 min) the aqueous phases were transferred into microfuge tubes containing fresh phenol, chloroform and isoamyl alcohol solution (300 μL)\(^1\). This step was repeated (3-5 times) until no more protein precipitation on the phenol-chloroform layers was observed. DNA solubility in the last aqueous phases was decreased by addition of 3 M (pH 4.8) sodium acetate (1/10 volume of the aqueous phase) and ice cold isopropanol (1 volume of aqueous phase). The mixtures were cooled (-20 °C) and after a period (48 h) the DNA was sedimented by centrifugation (13600xg, 5 min). The supernatants were discarded and the DNA pellets were washed with ice cold 70 % ethanol (500 μL). After further centrifugation (13600xg, 5 min) and elimination of the ethanolic supernatant, the DNA pellets were dried by warm incubation (60 °C, 15 min) of the open vials. The DNA was then dissolved in sterile demineralized water (50 μL) and stored (-20 °C) until further use.

\(^1\) A suitable mixture for DNA extraction constituted of phenol, chloroform and isoamyl alcohol in proportion 25:24:1, respectively, containing 10 mM Tris and 1 mM EDTA, pH 7.5-8.0, was purchased ready to use from ROTH.
4.4.1.2 Plasmids, amplification and extraction

Plasmids were used as DNA vectors for heterologous protein expression (pET derivatives) and for generation of deletion mutants (pK18mobsacB derivatives). The main properties of plasmids used in this study are summarized in table 2. They were isolated from *E. coli* DH5α, and stored in culture collections (if already existing). The newly constructed plasmids were specific derivatives, as described in the corresponding procedures (see below). Pure plasmid DNAs were stored frozen (-20 °C) or in *E. coli* DH5α in the culture collections.

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK18mobsacB</td>
<td>Kan', mob, sacB, ori pMB</td>
<td>Schäfer et al., 1994</td>
</tr>
<tr>
<td>pK18mobsacB_ΔdoeA (6672 bp)</td>
<td>Kan', pK18mobsacB with ΔdoeA fragment from <em>H. elongata</em></td>
<td>This work</td>
</tr>
<tr>
<td>pK18mobsacB::ΔdoeA (~7722)</td>
<td>Kan', pK18mobsacB with ΔdoeA fragment from <em>H. elongata</em></td>
<td>Schwibbert et al., 2011</td>
</tr>
<tr>
<td>pK18mobsacB_ΔeutB (7122 bp)</td>
<td>Kan', pK18mobsacB with ΔeutB fragment from <em>H. elongata</em></td>
<td>This work</td>
</tr>
<tr>
<td>pK18mobsacB::ΔeutC (7966 bp)</td>
<td>Kan', pK18mobsacB with ΔeutC fragment from <em>H. elongata</em></td>
<td>This work as Schwibbert et al., 2011.</td>
</tr>
<tr>
<td>pK18mobsacB_ΔectC (6714 bp)</td>
<td>Kan', pK18mobsacB with ΔectC fragment from <em>H. elongata</em></td>
<td>Witt, 2005</td>
</tr>
<tr>
<td>pET-15b</td>
<td>Amp', <em>T7lac</em>, N-(His)6-tag, thrombin cleavage site</td>
<td>Novagen (Madison/ WI, USA)</td>
</tr>
<tr>
<td>pET-15b::doeA -N-His</td>
<td>Amp', pET-15b with doeA from <em>H. elongata</em></td>
<td>This work</td>
</tr>
<tr>
<td>pET-22b(+)</td>
<td>Amp', <em>T7lac</em>, C-(His)6-tag, signal sequence for potential periplasmic localization.</td>
<td>Novagen (Madison/ WI, USA)</td>
</tr>
<tr>
<td>pET-22b doeA</td>
<td>Amp', pET-22b(+) with doeA from <em>H. elongata</em></td>
<td>This work</td>
</tr>
<tr>
<td>pET-22b_eutBC</td>
<td>Amp', pET-22b(+) with eutBC from <em>H. elongata</em></td>
<td>This work</td>
</tr>
</tbody>
</table>

For plasmid isolation, cells in an overnight culture (LB-1 medium, 4 mL) were pelleted (Biofuge fresco, Heraeus, 9500xg, 3 min) in one 2 mL capacity centrifugation vial. The supernatant was discarded and the cells were suspended (vortexing) in P1 buffer (50 mM Tris-HCl, 10 mM EDTA, 10 % w/v RNase A, pH 8.0; 200 μL). P2 buffer (200 mM NaOH, 1 % w/v SDS, pH 12.5, 200 μL) was added and the mixture was homogenized by careful vial inversion. This was followed by immediate addition of P3 buffer (3 M CH3COOK, pH 5.5; 200 μL) and further mixing by vial inversion (6 times) and centrifugation (16060xg, 3 min). The supernatant was then transferred to a clean microfuge tube with chloroform (500 μL). The mixture of liquids was strongly shaken and the insoluble substances sedimented from the upper layer by centrifugation (16060xg, 3 min). The DNA contained in the aqueous phase was rendered insoluble by separately mixing it with isopropanol (500 μL) in a clean microfuge tube followed by
strong shaking. DNA sample was recovered as a pellet after centrifugation (16060xg, 3 min) and elimination of the liquid phase. The DNA pellet was briefly washed by carefully addition of ice cold (70 % v/v) ethanol (500 μL) followed of short centrifugation (16060xg, 1 min) and removal of the supernatant. Plasmid DNAs isolated in this way were dried by placing the opened microfuge tubes in an oven (60 °C, 20 min). They were then dissolved in sterile demineralized water (50 μL) and stored (-20 °C) until further use.

4.4.2 DNA analysis by agarose gel electrophoresis

Agarose was mixed at a concentration of 1 % w/v (e.g. 0.3 g/30 mL) with 1xTAE buffer (40 mM Tris-HCl, 10 mM EDTA, 0.6 % v/v Acetic Acid, pH 8.0) and dissolved by briefly heating (near boiling) the solution. It was then allowed to cold down (55-65 °C) and poured into electrophoresis chambers (26 mL volume capacity; Horizon 58/GibcoBRL, Maryland, USA). Combs were placed into the agarose solution in the chambers. After this solution reached a gel consistency (>40 min), enough 1xTAE buffer to cover the gels was added. Gel casting accessories were then removed.

Samples to be analyzed were mixed with 6x loading buffer (60 mM EDTA, 0.09 % w/v bromophenol blue, 60 % v/v glycerol), and loaded into the corresponding wells. As a qualitative reference, a 1 kb marker or 100 bp marker plus (from Fermentas) was loaded into the gels. For estimation of DNA concentration a DNA mass ruler (from Fermentas) was also loaded into the gels. The separation of fragments was performed under an electric potential difference of 60-80 V (Electrophoresis Power Supply Consort E122, Sci.Plas Limited, Southham, UK).

For DNA detection, the gel was incubated in a solution of (1 % w/v) ethidium bromide (5 min) or in a gel red solution (20-30 min). The excess of the staining solution was removed by incubating the gels in demineralized water (>7 min). UV-profiles of DNA detected on gels were then photographed (Videosystem Intas, Göttingen). In cases where further DNA purification was required, the gel sections containing the DNA bands of interest were cut off with a scalpel blade, transferred into a clean microfuge tube and treated as described in section 4.4.3.3.1.
The concentration of DNA in solutions was estimated by comparing the intensity of bands due to ethidium bromide (or gel red) detection in agarose gels with those generated by a DNA standard mixture of known DNA composition (DNA mass ruler). Concentrations estimated in this way (ng/µL) were expressed in terms of mol assuming that 1 bp has a mass of 660 daltons, and therefore, for a given fragment: \( \text{MW}_{g/mol} = \text{fragment size}_{bp} \times 660 \).

4.4.3 Enzymatic DNA modification

4.4.3.1 DNA amplification by polymerase chain reaction (PCR)

PCR is used for the enzymatic amplification of specific DNA sections (target DNA) from cloned or genomic DNA sequences (template DNA). The DNA polymerases catalyze the step by step addition of deoxyribonucleotide units (dNTPs) to the 3'OH end of an oligonucleotide primer. This primer is appropriately designed so that the elongated DNA polymer (5'→3') yielded as a product complements the single strand DNA used as a template. Depending on the purpose of the amplification, polymerases with different degrees of reliability can be used. Important information about the DNA polymerases used in this study was extracted from the user protocols provided by the manufacturer and is summarized in table 3.

Table 3. DNA polymerases used in this research.

<table>
<thead>
<tr>
<th>Name</th>
<th>Proofreading activity</th>
<th>Units for 50 µL reaction volume</th>
<th>Elongation time</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq DNA Polymerase (recombinant)</td>
<td>ND (^a)</td>
<td>1.25</td>
<td>1 min/Kb</td>
<td>Fermentas</td>
</tr>
<tr>
<td>5 primer Taq DNA Polymerase</td>
<td>ND (^a)</td>
<td>1.25</td>
<td>1 min/Kb</td>
<td>5 primer</td>
</tr>
<tr>
<td>KOD DNA Polymerase</td>
<td>yes</td>
<td>1.25-2.5</td>
<td>2 min/Kb</td>
<td>5 primer</td>
</tr>
<tr>
<td>Pfu DNA Polymerase</td>
<td>yes</td>
<td>1.25-2.5</td>
<td>2 min/Kb</td>
<td>Fermentas</td>
</tr>
<tr>
<td>Phusion™ DNA Polymerase</td>
<td>yes</td>
<td>0.5-1.0</td>
<td>0.25-0.5 min/Kb</td>
<td>Finnzymes</td>
</tr>
</tbody>
</table>

\(^a\)ND=Not detected

For genetic engineering and sequencing, high fidelity DNA polymerases with proofreading activity were used. Depending on further applications of the DNA product, DNA amplification can be done with one primer (e.g. sequencing), two (e.g. gene
cloning) or more. Some primers can be designed to include base mutations or restriction sites as long as they are complementary to the templates and the annealing temperature is appropriate (Lodge et al., 2007). The main properties of the primers used in this study are presented in table 4. They were obtained dried from Eurofins MWG Operon (Ebersberg, Germany), dissolved in sterile demineralized water and stored frozen (-20 °C).

Table 4. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Target DNA</th>
<th>DNA sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tm (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>He3665F1</td>
<td>doeA</td>
<td>GAGCGA&lt;sub&gt;c&lt;/sub&gt;GTAG&lt;sub&gt;3&lt;/sub&gt;ATTCAAGITTTTCACTAC</td>
<td>63.9</td>
<td>This work</td>
</tr>
<tr>
<td>He3665R2</td>
<td>doeA</td>
<td>CGGA&lt;sub&gt;AG&lt;/sub&gt;GCTT&lt;sub&gt;1&lt;/sub&gt;GCTCATGTTGACTC</td>
<td>66.1</td>
<td>This work</td>
</tr>
<tr>
<td>He3665R3</td>
<td>doeA</td>
<td>GCTTGA&lt;sub&gt;T&lt;/sub&gt;ATG&lt;sub&gt;3&lt;/sub&gt;GTTGACCTTTAAGTTGAC</td>
<td>65.1</td>
<td>This work</td>
</tr>
<tr>
<td>He3660F1</td>
<td>eutBC</td>
<td>GAGGT&lt;sub&gt;C&lt;/sub&gt;A&lt;sub&gt;T&lt;/sub&gt;AG&lt;sub&gt;3&lt;/sub&gt;TCAAGGGCTGCAC</td>
<td>64.4</td>
<td>This work</td>
</tr>
<tr>
<td>He3660R2</td>
<td>eutBC</td>
<td>ATCC&lt;sub&gt;A&lt;/sub&gt;GCT&lt;sub&gt;T&lt;/sub&gt;CTGGTAAGGCTGCCTAC</td>
<td>66.5</td>
<td>This work</td>
</tr>
<tr>
<td>SOE PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>He3655SOE-F1</td>
<td>doeA&lt;sub&gt;_up&lt;/sub&gt;F</td>
<td>GGACGG&lt;sub&gt;a&lt;/sub&gt;ATTC&lt;sub&gt;G&lt;/sub&gt;GGAATTGCGCAGATAGG</td>
<td>68.1</td>
<td>This work</td>
</tr>
<tr>
<td>He3655SOE-R1</td>
<td>doeA&lt;sub&gt;_down&lt;/sub&gt;R</td>
<td>TCA&lt;sub&gt;T&lt;/sub&gt;T&lt;sub&gt;a&lt;/sub&gt;GTTGGA&lt;sub&gt;G&lt;/sub&gt;GCCGCGAAC</td>
<td>70.7</td>
<td>This work</td>
</tr>
<tr>
<td>He3660SOE-F2</td>
<td>eutB&lt;sub&gt;_up&lt;/sub&gt;F</td>
<td>GCA&lt;sub&gt;G&lt;/sub&gt;G&lt;sub&gt;A&lt;/sub&gt;TTCC&lt;sub&gt;T&lt;/sub&gt;GTGGACCTACTACGG</td>
<td>68.1</td>
<td>This work</td>
</tr>
<tr>
<td>He3660SOE-R2</td>
<td>eutB&lt;sub&gt;_down&lt;/sub&gt;R</td>
<td>GAGGGGATTG&lt;sub&gt;G&lt;/sub&gt;GACTCGCTGCGCCATTGAGG</td>
<td>68.1</td>
<td>This work</td>
</tr>
<tr>
<td>He3665SOE-R1</td>
<td>eutB&lt;sub&gt;_down&lt;/sub&gt;R</td>
<td>TCCAGTT&lt;sub&gt;T&lt;/sub&gt;Aa&lt;sub&gt;g&lt;/sub&gt;CT&lt;sub&gt;T&lt;/sub&gt;CTGGGGGTCTGCGG</td>
<td>69.5</td>
<td>This work</td>
</tr>
<tr>
<td>He366'0F1</td>
<td>eutC&lt;sub&gt;_up&lt;/sub&gt;F</td>
<td>CCGT&lt;sub&gt;G&lt;/sub&gt;G&lt;sub&gt;A&lt;/sub&gt;TTCC&lt;sub&gt;T&lt;/sub&gt;CTGGGGGTCTGCGG</td>
<td>59.8</td>
<td>Schwibbert, K.</td>
</tr>
<tr>
<td>He365'8R1</td>
<td>eutC&lt;sub&gt;_down&lt;/sub&gt;R</td>
<td>CGGCC&lt;sub&gt;C&lt;/sub&gt;ATTCAAG&lt;sub&gt;C&lt;/sub&gt;TAACACTG</td>
<td>60.2</td>
<td>Schwibbert, K.</td>
</tr>
<tr>
<td>Screening&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>He2590F1</td>
<td>ΔectC</td>
<td>ATATCGCGCAAGACTACC</td>
<td>57.3</td>
<td>This work</td>
</tr>
<tr>
<td>He2590R1</td>
<td>ΔectC</td>
<td>TACT&lt;sub&gt;G&lt;/sub&gt;GATTCCGCGTTAGAC</td>
<td>56.7</td>
<td>This work</td>
</tr>
<tr>
<td>He366'0F2</td>
<td>Δeuc&lt;sub&gt;T&lt;/sub&gt;C</td>
<td>GACA&lt;sub&gt;A&lt;/sub&gt;ATCGC&lt;sub&gt;G&lt;/sub&gt;ACCCCTTC</td>
<td>58.8</td>
<td>This work</td>
</tr>
<tr>
<td>He365'8R2</td>
<td>Δeuc&lt;sub&gt;T&lt;/sub&gt;C</td>
<td>CGATCGG&lt;sub&gt;T&lt;/sub&gt;GATGCTCGACG</td>
<td>58.8</td>
<td>This work</td>
</tr>
<tr>
<td>He366'6F1</td>
<td>ΔdoeA</td>
<td>TGGCGGTA&lt;sub&gt;T&lt;/sub&gt;ATTG&lt;sub&gt;A&lt;/sub&gt;TGCAAGG</td>
<td>57.3</td>
<td>This work</td>
</tr>
<tr>
<td>He366'4R1</td>
<td>ΔdoeA</td>
<td>CCACC&lt;sub&gt;C&lt;/sub&gt;GTACAGGAAATCATC</td>
<td>59.3</td>
<td>This work</td>
</tr>
<tr>
<td>RACE ectA&lt;sub&gt;F&lt;/sub&gt;</td>
<td></td>
<td>GCC&lt;sub&gt;T&lt;/sub&gt;T&lt;sub&gt;T&lt;/sub&gt;C&lt;sub&gt;T&lt;/sub&gt;GTAACAAGACCTG</td>
<td>57.3</td>
<td>Schwibbert, K.</td>
</tr>
<tr>
<td>RACE ectA&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td>ACTTGACCA&lt;sub&gt;G&lt;/sub&gt;CCTGATAGT</td>
<td>57.3</td>
<td>Schwibbert, K.</td>
</tr>
<tr>
<td>Sequencing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13rev(-48)</td>
<td>pK18mobsacB</td>
<td>AGCGGATAA&lt;sub&gt;C&lt;/sub&gt;ACAAATTTCAC</td>
<td>49.1</td>
<td>Eurofins</td>
</tr>
<tr>
<td>M13for(-47)</td>
<td>pK18mobsacB</td>
<td>CCAGGGT&lt;sub&gt;T&lt;/sub&gt;T&lt;sub&gt;T&lt;/sub&gt;CCAGCTC</td>
<td>55.2</td>
<td>Eurofins</td>
</tr>
<tr>
<td>Seq3658-F2</td>
<td>eutC&lt;sub&gt;_down&lt;/sub&gt;</td>
<td>CTCT&lt;sub&gt;T&lt;/sub&gt;CGACGCTGAGT</td>
<td>56.0</td>
<td>This work</td>
</tr>
<tr>
<td>Seq3660-F2</td>
<td>eutC&lt;sub&gt;_up&lt;/sub&gt;F</td>
<td>CGT&lt;sub&gt;G&lt;/sub&gt;GAGTG&lt;sub&gt;G&lt;/sub&gt;ATGGAAGA</td>
<td>53.7</td>
<td>This work</td>
</tr>
<tr>
<td>T7</td>
<td>pET</td>
<td>TAAATCA&lt;sub&gt;G&lt;/sub&gt;ACTCTTATATAGG</td>
<td>50.2</td>
<td>Eurofins</td>
</tr>
<tr>
<td>T7-terminator</td>
<td>pET</td>
<td>GCTAGTT&lt;sub&gt;T&lt;/sub&gt;ATGCTCAACGG</td>
<td>56.0</td>
<td>Eurofins</td>
</tr>
<tr>
<td>Seq3659-F2</td>
<td>eutC</td>
<td>AGGCCGCGTGGTGATATCGT</td>
<td>56.0</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lowercase letters indicate base modification required for primer optimization. Restriction sites are underlined and restriction enzymes are shown as superscripts letters in the sequences: E=EcoR1, N=NdeI, H=HindIII

<sup>b</sup> Screening for deletion mutants was sometimes performed with up and down primers used in SOE-PCR.

Jhonny Correa: Hydroxyectoine metabolism in Halomonas elongata
The basic components in a mixture to be subjected to PCR are illustrated in table 5. Before the reaction can commence, the template DNA is denatured (>93 °C; 0.5-5 min) to form two complementary single strands of DNA. The reaction mixture is quickly cooled down to the appropriate temperature (50-72 °C) to prevent immediate reannealing of long DNA strands and allow annealing of the primers, which are provided in excess. This temperature is maintained for a short time (10-60 s). Due to their small size, primers anneal rapidly and specifically to their complementary sequences. Further increase of the temperature (usually to ≈72°C) is performed to match the optimum condition for DNA polymerase to catalyze the successive addition of deoxyribonucleotide triphosphates (dNTPs, provided in excess). This reaction can be of variable velocity depending on the enzyme. The period to complete the elongation (15 s - 5 min) will also depend on the length of the fragment to be amplified (Lodge et al., 2007). An example of a typical temperature cycling for PCR is shown in table 6.

### Table 5. Components in a mixture for PCR using Phusion DNA polymerase

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume in 50 μL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>21.5 μL</td>
<td></td>
</tr>
<tr>
<td>Phusion HF Buffer (5x)</td>
<td>10.0 μL</td>
<td>1x*</td>
</tr>
<tr>
<td>dNTPs (2 mM, fresh)</td>
<td>5.0 μL</td>
<td>200 μM each*</td>
</tr>
<tr>
<td>Forward primer (50 pmol/μL)</td>
<td>1.0 μL</td>
<td>0.2-1.0 μM* / 1 μM</td>
</tr>
<tr>
<td>Reverse primer (50 pmol/μL)</td>
<td>1.0 μL</td>
<td></td>
</tr>
<tr>
<td>Betaine (5 M)</td>
<td>10.0 μL</td>
<td>1 M</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0 μL</td>
<td>1 pg-10 ng*</td>
</tr>
<tr>
<td>Phusion DNA polymerase (2U/μL)</td>
<td>0.5 μL</td>
<td>0.02 U/μL*</td>
</tr>
</tbody>
</table>

*Recommended by the manufacturer

### Table 6. Temperature cycling during a typical PCR using Phusion DNA polymerase

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98.0 °C</td>
<td>50 s</td>
</tr>
<tr>
<td>[30 times] Amplification cycles</td>
<td>98.0 °C</td>
<td>10 s (denaturation)</td>
</tr>
<tr>
<td></td>
<td>71.0 °C</td>
<td>30 s (annealing)</td>
</tr>
<tr>
<td></td>
<td>72.0 °C</td>
<td>15 s (elongation)</td>
</tr>
<tr>
<td>End cycle</td>
<td>72.0 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>Hold/storage temperature</td>
<td>4.0 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

4.4.3.1.1 Splicing by overlap extension (SOE) PCR.

PCR is also used in the precise *in vitro* synthesis of DNA sequences suitable for gene deletion through a variety of genetic engineering techniques involving DNA recombination (section 4.4.7). For gene deletion, sequences upstream and downstream of the target genes (*doeA*, *eutB* and *eutC*) were amplified by PCR using...
the primers indicated in table 4. Restriction sites were included in the primers annealing at the farther (from the target gene) region of the amplified sequences whereas each primer at the nearer side was formed by homologous sequences both upstream and downstream of the target genes. The appropriate flanking regions (PCR amplified) were then joined together, leading to a splicing by overlap extension (SOE) PCR technique (Horton et al., 1989). The resulting PCR fragments, with the appropriate restriction sites, were ligated into the shuttle vector pK18mobsacB (section 4.4.3.2).

4.4.3.1.2 In situ PCR

The DNA to be used as a template in PCR can also be added when still in the cells. This is advantageous because it allows PCR to be used to evaluate cells reproduced after genetic manipulation, and thus to identify colonies containing specific DNA sequences. That is, it possible to screen for deletions mutants or for cells containing plasmids with DNA insertions. In this screening method, a small amount of biomass from a colony is taken with a sterile wooden stick. It is then resuspended in sterile demineralized water (30-50 μL) and some of the suspension (≈0.5 μL, even one cell might be enough) is added to the PCR mixture, which is still lacking DNA polymerase. The cells are then disrupted by heating the PCR mixture (95 °C, 10 min). After cooling the mixture, the polymerase is added in order to proceed with the appropriate thermal cycling for DNA amplification. As the purpose of this method is for screening only, DNA polymerase without proofreading activity were used.

4.4.3.2 Enzymatic digestions

4.4.3.2.1 Construction of vectors

Restriction enzymes (e.g. Ndel, HindIII) were used to cut double stranded DNA in order to generate sticky ends that could be later joined in order to construct vectors. For the digestion procedure considered the recommendations of the enzyme manufacturer for the enzymes.

*Enzyme restriction and first dephosphorylation.* The reaction setup was adjusted for mixtures in a total volume of 40 μL which contained: 10 μL of pure DNA (vector or insert), 4 μL of buffer, 2 μL (20 U) of restriction enzyme and 24 μL of sterile
demineralized water. Restriction digestion was performed by incubation (37 °C, >2 h) in a thermocycler. After 1.5 h, further digestion of vector DNA (>30 min) was allowed in the presence of alkaline phosphatase (Fast-AP, 1 μL, 1 U). The restricted DNA was then subjected to purification (section 4.4.3.3.1) and the concentration of the pure DNA was estimated on agarose gel (section 4.4.2).

**Second dephosphorylation and ligation.** Pure restricted vectors (20-50 ng) were subjected to a second dephosphorylation (Fast-AP, 1 μL, 1 U) by incubating (37 °C, 20 min) the buffered reaction mixture (<10 μL) in a thermocycler. The reaction was stopped by heat inactivation (75 °C, 5 min) of the Fast-AP in the reaction mixture which was then cooled down (on ice). Appropriate volumes to complete the total volume of 20 μL were added, including: buffer, T4-DNA ligase (1 μL, 1 U), insert DNA (1:3 mass ratio vector/insert) and water. Sticky DNA ligation was allowed by incubation (22 °C, >10 min) of the solution in a thermocycler. T4-DNA ligase in the reaction mixture was heat-denatured (70 °C, 5 min), and then the mixture containing the ligation products was used to transform competent cells of *E. coli* DH5α (section 4.4.5).

### 4.4.3.2.2 Diagnostic of plasmid identity

For the digestion procedure, the recommendations of the enzyme manufacturer were followed. Reaction mixtures were adjusted to a total volume of 20 μL and contained: 2 μL of pure DNA (vector), 2-4 μL of buffer (to 1-2x), 1-2 μL (10-20 U) of restriction enzyme and sterile demineralized water (to 20 μL). Restriction digestion was performed by incubation (37 °C, >1 h) in a thermocycler. The restriction reaction was then evaluated on an agarose gel (section 4.4.2). Examples of enzymes used for the evaluation of the plasmids constructed in this work are listed in table 7.

<table>
<thead>
<tr>
<th>Plasmid (size in bp)</th>
<th>Enzymes (buffer)</th>
<th>Fragments yielded (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK18mobsacB ΔdocA (6672)</td>
<td>EcoRV, NcoI (Tango)</td>
<td>1635, 5037</td>
</tr>
<tr>
<td>pK18mobsacB ΔeutB (7122)</td>
<td>Alw44I (Tango)</td>
<td>1983, 5139</td>
</tr>
<tr>
<td>pK18mobsacB::ΔeutC (7966)</td>
<td>SmaI (Tango)</td>
<td>842, 7124</td>
</tr>
<tr>
<td>pET-22b docA (6597)</td>
<td>EcoRV (R)</td>
<td>2193, 4404</td>
</tr>
<tr>
<td>pET-22b eutBC (7398)</td>
<td>EcoRV (R)</td>
<td>2792, 4666</td>
</tr>
</tbody>
</table>

Jhonny Correa: Hydroxyectoine metabolism in *Halomonas elongata*
4.4.3.3 Purification of DNA products after enzymatic reactions

4.4.3.3.1 DNA purification after agarose electrophoresis
Enzymatic reaction products containing more than one DNA polymer (i.e. PCR products and plasmid restriction products for directional cloning) were submitted to agarose gel electrophoresis (section 4.4.2). The gel portions containing the target DNA were cut off from the gel and placed into microfuge tubes. Further purification of the DNA was performed with the material of a Peqlab gel extraction kid (peqGOLD gel extraction kit, Peqlab, Erlangen) according to the manufacturer’s instructions. This included the dissolution of the DNA-containing gel by heating (60-65 °C) in the presence of a buffer, followed of separation by affinity to silica membranes. The elution of the DNA was performed with sterile demineralized water (30-50 μL).

4.4.3.3.2 Direct DNA purification from enzymatic reactions
Products of enzymatic reactions of one main DNA product (i.e. plasmid restriction with a single cutter) were purified with the same gel extraction kid mentioned above (peqGOLD gel extraction kit, Peqlab, Erlangen). This time, however, the agarose gel electrophoresis step was skipped and so, the reaction solution was just mixed directly with the appropriate buffer (1:1), and then the buffered mixture was applied to the silica membrane.

4.4.4 Preparation of competent cells

Cells in an overnight culture (700 μL) were subcultured in 2x YT medium (70 mL). Growth was allowed (30°, 180 rpm) until the culture reached a density known to contain cells rapidly dividing (OD 600 nm ≈ 0.4). At this point, the culture was separated into two portions (=35 mL each) to harvest the cells by centrifugation (4000 rpm, 4 °C, 6 min). The liquid phase was decanted and competence was induced by suspension of the cells pellets in an ice cold CaCl2/MgSO4 solution (70/20 mM ratio2; 5.5 mL/pellet) followed by incubation (on ice, 30 min) to allow cells to interact with Ca2+ and Mg2+ ions. Afterwards, the suspension was centrifuged (4000 rpm, 4 °C, 10 min) and the liquid

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2 50 mL (70/20 CaCl2/MgSO4): 3.5 mL of 1 M CaCl2+1mL of 1 M MgSO4+45.5 mL water.
Material and Methods

Phase was removed by decantation. Enhancement of the permeability of the cell envelope to DNA was further achieved by a second suspension of pellets in an ice cold CaCl₂/MgSO₄ solution (70/20 mM ratio; 1.8 mL/pellet) and incubation (on ice, 30 min). Cells in the competent state were exposed to glycerol (ice cold, 875 µL) for better survival after frozen storage (-70 °C) as cell aliquots (200 µL each) (Vielgraf, 2008; Pühler and Timmis, 1984).

4.4.5 Transformation

Competent cells (200 µL) were thawed on ice (≈10 min incubation). The DNA solution, i.e. ligation products (20 µL, <100 ng of DNA) or pure plasmid (0.5 µL), was added, followed by gentle mixing of the solution by swirling with a pipette tip. The interaction of the DNA molecules with lipopolysaccharides at the outer surface of cells was allowed while the sample stayed on ice (20-30 min). The uptake of properly located ligation product at the membranes was further induced by a heat shock resulting from incubation in a water bath (42 °C, 90s) followed immediately by ice (1-2 min). 2x YT medium (0.5 mL/200 µL of transformed cells) was added to provide nutrients for the cells to recover during an incubation period (37 °C; 45-60 min) during which overexpression of antibiotic resistance took place. Media with recovered cells (200 µL) were transferred to LB-1 agar plates containing the appropriate antibiotic. Propagation of clones to form colonies was promoted by incubation at 37 °C for 16-20 h (Vielgraf, 2008; Pühler and Timmis, 1984; Lodge et al., 2007). First verification of the plasmid in the clones was deduced by in situ PCR (section 4.4.3.1.2). Additionally, the clones were cultured, the plasmid isolated (section 4.4.1.2) and digested (section 4.4.3.2.2) or sequenced (section 4.4.6).

4.4.6 DNA sequencing

Sequences inserted in the constructed vectors were determined following the didesoxy method (Sanger et al., 1977) by the company SequiServe (Vaterstetten, Germany) with the primers specified for such a purpose in table 4.
4.4.7 Deletion of genes by double recombination events

4.4.7.1 Plasmid conjugation and selection of cells with integrated plasmid

*E. coli* S17-1 was separately transformed with vector pK18mobsacB derivatives carrying the appropriate sequences to delete doeA, ectC or eutC. Plasmid transfer to the specific *H. elongata* recipient strains was promoted by inducing conjugation. For this, both the donor strains (*E. coli* S17-1 pK18mobsacB, in agar plates of AB-2 kan100) and *H. elongata* recipient strains (in AB-1, 50 mL) were grown in parallel overnight cultivations (37 °C). Cells of donor strains were then separately suspended in pre-warmed AB-1 medium (5 mL each). Appropriate volumes of cell suspensions of donors and cultures of the recipient strains were mixed as to produce cell suspensions in a 1:4 biomass ratio (donor: recipient), estimated from the corresponding optical cell densities. Cell mixtures in suspensions were pelleted by centrifugation (5000xg, 2 min). Supernatants were decanted and the cell pellets were suspended in their remaining liquid. The suspensions were then separately applied on top of sterile filter papers laid over pre-warmed (37 °C) AB-1 agar medium in plates. These plates were further incubated (37 °C, overnight) to promote bacterial plasmid DNA exchange and plasmid integration into the genome by homologous recombination (*single cross-over*). The mixture of cells propagated over the different filters were taken with a scalpel blade and separately suspended in sterile NaCl solutions (2 % w/v, 900 µL each). These were then used to separately inoculate MM63-3 kan250 media (50 mL each) where only *H. elongata* strains with plasmid integrated into their chromosome could reproduce during the incubation period (30 °C, 2-4 days). In order to enhance the selectivity of *H. elongata* strains with recombined plasmids, dilution series (10⁻¹-10⁻⁶) in sterile (2 % w/v) NaCl solution were prepared from each culture in minimal medium. Aliquots (100 µL of each) were then streaked (by using a sterile inoculation loop) across the surface of MM63-3 kan250 agar plates, and these were further incubated (30 °C, 2-5 days).

4.4.7.2 Gene deletion and selection of mutants

A *double cross-over* involving both flanking regions of the target genes was promoted in cells from occurring in single colonies with integrated plasmids (section 4.4.7.1). This was done by transferring cell material from colonies to LBG-2 medium (50 mL), followed of overnight cultivation (30 °C). Culture dilutions (10⁻¹-10⁻⁶) were then prepared and
(100 μL of each) streaked (by using a sterile inoculation loop) across the surface of plates of LBG-2 agar containing sucrose (22 % w/v). Selection of arising strains without plasmid DNA\textsuperscript{3} was accomplished by further cultivation (37 °C, 16-24 h). Screening for deletion mutants in arising single colonies was performed by \textit{in situ} PCR (section 4.4.3.1.2). From colonies that were positive to target deletions, mutant cells were selected and seeded in AB-5 agar plates from which growing cells were subsequently submitted to culture collections and used for further experiments.

4.5 Characterization of \textit{Halomonas elongata} strains

4.5.1 Growth assays

Cells in overnight cultures of \textit{H. elongata} WT, \textit{ΔdoeA, ΔdoeB, ΔeutB, ΔeutC, ΔectC, ΔdoeAΔectC, ΔdoeAΔeutC, ΔeutBΔectC, ΔeutBΔectCΔdoeA, ΔeutBΔdoeA, ΔectA, ΔectAΔdoeA, ΔectB, ΔectR and ΔeutBΔeutCectB::ΩΔdoeD} in MM63-3 medium were harvested by centrifugation (4000 rpm, 3 min). The supernatants were removed and cells were suspended in a volume of 2 % NaCl necessary to produce suspensions with a photon absorption equal to 2. These suspensions (20 μL per well) were used for the inoculation of MM63-6 medium (200 μL per well) in 96 wells plates containing either: (i) only glucose as a carbon source (1 mM), (ii) ectoine as an additional carbon source (10 mM), or (iii) hydroxyectoine (10 mM). To test the phenotypic impact of the mutations, the cells were cultivated in a multi mode microplate reader (Synergy\textsuperscript{TM} HT, BioTek\textregistered Instruments, Inc. Vermont, USA) with shaking (stopping 5 min before growth measurements). Cells were maintained at 30 °C and monitored for photon absorption (600 nm) every 30 min. Growth on glucose was performed in duplicates, while growth in glucose-ectoine or glucose-hydroxyectoine was performed in triplicates. The experiments were repeated twice and the results were similar.

4.5.2 Hydroxyectoine conversion to ectoine

Overnight cultures of strains \textit{H. elongata} WT, \textit{ΔdoeA, ΔdoeB, ΔeutB, ΔeutC, ΔectC, ΔdoeAΔectC, ΔdoeAΔeutC, ΔeutBΔectC, ΔeutBΔectCΔdoeA, ΔeutBΔdoeA, ΔectA and ΔectAΔdoeA}, processed as above, were also used (100 μL of each) for the inoculation

\textsuperscript{3} Cells carrying the plasmid pK18mobsacB derivatives are able to express a protein called levansucrase, which catalyze sucrose polymerization and therefore, these cells are killed during growth on sucrose containing media due to cytoplasm clotting.
of Bp-11 medium (25 mL in 100 mL capacity Erlenmeyer flasks) in order to analyze their ability to convert hydroxyectoine in ectoine. The cultivation was performed at 30 °C (120 rpm) and the biomass growth was monitored by reading the photon absorption (595 nm) of 200 μL of each culture (or its dilution) in a microplate reader. Samples for quantification of ectoines and total protein were taken (sections 4.6.1) and extracted (4.6.2 and 4.7.1, respectively). Quantification of ectoines was determined at BAM according to Schwibbert et al. (2011) and proteins were quantified for normalization of data, according to section 4.7.2.

### 4.5.3 Screening for intermediate accumulation during degradation of ectoines

Overnight cultures in MM63-3 medium (3 mL each, in 15 mL capacity sterile test tubes) of *H. elongata* ΔeutC, ΔectC, ΔdoeAΔectC, ΔdoeAΔeutC, ΔeutBΔectC, ΔeutBΔectCΔdoeA, ΔeutBΔdoeA, ΔectA, ΔectR, ΔeutBΔeutCectB::ΩΔdoeD and ΔectAΔdoeA were used for inoculation of MM63-6 medium containing as a carbon source either: (i) a mixture of 5 mM glucose and 15 mM ectoine, or (ii) a mixture of 5 mM glucose and 15 mM hydroxyectoine (20 mL each, in 100 mL capacity Erlenmeyer flasks). Cultivation was performed at 30 °C (180 rpm) and bacterial growth was monitored with a photometer. Cells in 1-2 mL cultures were harvested from cultures with optical density above 0.8 for subsequent extraction of intracellular small polar metabolites and analysis of their molecular composition by HPLC (section 4.6.1-4). This experiment was scaled up to 0.25 L as described in section 4.6.3.

### 4.6 Analysis of small polar metabolites

#### 4.6.1 Harvest of biomass

Cells in culture samples (1-2 mL, OD>0.5) were harvested by centrifugation (10000xg, 3 min). The supernatants were transferred to clean microfuge tubes and stored (-20 °C) as media samples. The remaining parts of the liquid phases were carefully removed and biomass pellets were submitted to a quick washing step with isotonic NaCl solution (0.25 mL). After centrifugation (10000xg, 3 min) and discarding of the liquid phase, the fresh biomass was stored (-20 °C), either for compatible solute extraction or total protein quantification (sections 4.6 and 4.7.1-2, respectively).
4.6.2 Extraction of polar metabolites

Lysis of cells in fresh biomass pellets (see above) was accomplished by suspension of the cells in 500 μL of a mixture of methanol, chloroform and water in a ratio of 10:5:4 by volume, respectively. Dissolution of the membrane and cytoplasmic extraction was facilitated by shake incubation (RT, >1 h). Separation of organic and aqueous phases was induced by the addition of chloroform (130 μL) and water (130 μL), thus leading the molecules to a solvent fractionation. To help the distribution of components between the phases to reach equilibrium, samples were further incubated with shaking (RT, >15 min). Clear definition of phases was obtained by centrifugation (10000xg, 10 °C, 10 min) and the aqueous phases (~500 μL) were carefully transferred to clean microfuge tubes and stored (-20 °C). Methods were modified from Bligh and Dyer (1959).

4.6.3 Up-scaling of strain Halomonas elongata K2.3 (ΔeutBΔeutCectB::ΩΔdoeD)

Up-scaling of strain H. elongata K2.3 (ΔeutBΔeutCectB::ΩΔdoeD) was accomplished by reproducing the experiments outlined in section 5.4.3 in 0.25 L, but increasing the concentration of hydroxyectoine from 15 mM to 20mM. The harvested cells (OD\textsubscript{600 nm} =~1.650.25 h cultivation) were immediately extracted using 10 mL of a solution of methanol, chloroform and water in a ratio of 10:5:4 by volume, respectively. To this mixture were added 2.6 mL of chloroform and 2.6 mL of water. After centrifugation (10 min, 15000 rpm), the aqueous phase was transferred to a clean falcon tube (50 mL capacity) and the liquids were evaporated by heating (60°C, 36 h) in an oven yielding 82,5 mg of dry material. The composition of extracted metabolites was analyzed by NMR.

4.6.4 Synthesis of potential intermediates through hydrolysis of ectoines

Hydrolysis as enzyme substrate. Ectoines were separately subjected to alkaline hydrolysis with KOH (Kunte et al., 1993). Hydrolysis of pure ectoine (50 mM) or hydroxyectoine to yield mixtures of the corresponding α and γ NADA derivatives was achieved in 10 mL of 0.1 M KOH by incubation at 50°C. The reaction was stopped after 20 h by neutralization with concentrated HCl.
**Material and Methods**

**Hydrolysis products as NMR standards.** Hydroxyectoine (25 g) was dissolved in water (0.25 L) and NaOH (5 g) was added. The pH was adjusted (to 12.4), and the mixture was incubated with stirring (RT) and regular pH adjustment (to 12.4, RT) until residual hydroxyectoine dropped below 2% (four days). The reaction was then stopped by neutralization and molecules with a net charge at pH 7 were separated by electrodialysis. The salt free solution was frozen and water was removed by sublimation in a freeze dryer.

4.6.5 **High performance liquid chromatography (HPLC)**

The composition of cell extracts with small polar metabolites (section 4.6.2) was evaluated by HPLC in a system equipped with a Spectra System SCM1000 degasser (Thermo Scientific), a Spectra System P100 pump (Thermo Separation Products), a Rheodyne injector (20 μL, Nr. 7125, Rheodyne Inc.), and a LiChrospher 100-NH2 precolumn (5 μm, Merck). Separation of different compounds was accomplished on a Grom-Sil Amin-1PR column (125x4 mm, 3 μm, LiChrocart-System, Alltech Grom GmbH) eluting isocratically with 80 % (v/v) acetonitrile at a flow rate of 1 mL/min. The elution of compounds was monitored by tracking the UV absorption (λ=210 nm; Spectrasystem UV 1000, Thermo Separation Products) of the eluted mobile phase. Quantification of ectoines was accomplished by comparing the area of the corresponding peak in the HPLC profiles (Software ChromQuest 4.2.34, Version 3.1.6, Thermo Quest Cooperation) with areas observed for known standards.

The composition of amino reactive compounds (molecules with primary and secondary amine groups) was analyzed by HPLC after derivatization with FMOC (see below). In this case the HPLC system was equipped with a Spectra System SCM1000 degasser (Thermo Scientific), a Spectra System P2000 pump (Thermo Separation Products), a Spectra System AS3000 Autosampler (Thermo Separation Products), a LiChrospher 100-NH2 precolumn (5 μm, Merck). Compound separation was accomplished on a Merck Superspher 60 RP-8 column (125x4 mm, 4 μm; LiChrocart-System, Alltech Grom GmbH), eluting with a stepwise gradient of solution A and solution B at a flow rate of 1.25 mL/min (Table 8). The elution of compounds was monitored by tracking the UV absorption (λ=316 nm) of the eluted mobile phase. Chromatograms were assessed with ChromQuest 4.2.34 software, Version 3.1.6 (Thermo Quest Cooperation).
Table 8. HPLC gradient of solutions to elute compounds in samples after FMOC derivatization.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution A(^a) (%)</th>
<th>Solution B(^b) (%)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>60</td>
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</tr>
<tr>
<td>4</td>
<td>0</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
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<td>100</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>0</td>
<td>49</td>
</tr>
</tbody>
</table>

\(^a\) Solution A: 1:40:160 volume proportions of THF, CH\(_3\)CN and 50 mM-pH 4.2 CH\(_3\)COONa, respectively

\(^b\) Solution B: 4:1 volume proportions of CH\(_3\)CN and 50 mM-pH 4.2 CH\(_3\)COONa, respectively

**FMOC derivatization** (modified after Kunte et al., 1993). This chemical modification has been used to facilitate the detection of nitrogen-containing compounds with little UV activity by covalently linking the free amino groups of molecules in the samples to a fluorenyl-metoxycarbonyl group (\(\lambda_{\text{max}}=316\) nm). For this, an internal standard (0.25 mM norvaline or taurin in \(\approx 0.50 \text{ M borate buffer pH 7.7; 40 \mu L}\) was included in the sample (40 \(\mu\)L, diluted if necessary) as the pH of the solution was alkalinized and buffered. The FMOC reagent (1 mM FMOC in acetone; 80 \(\mu\)L) was added and the condensation reaction allowed to proceed (45 s) with shaking (IKA Vibrax). The excess of FMOC reagent was then inactivated by shaking (45 s) in the presence of an excess of ADAM (40 mM ADAM in a solution of 50 % v/v acetone in 0.25 M borate buffer (pH 7.7; 100 \(\mu\)L). The mixtures with the reaction products were diluted in HPLC mobile phase (solution A, see table 8) and then analyzed by HPLC, as described above.

**Note.** In the FMOC HPLC profiles herein reported, highly concentrated samples were subjected to derivatization. It could be that in some samples FMOC was the limiting reagent and therefore any quantitative interpretation of these results might be subject to considerable errors (>5 %). Nevertheless the modification in the concentration has the advantage of producing relatively small [unwanted FMOC derivative signals](#) (about 23.4, 25.8 and 45.0 min), raising the significance of small peaks.

**OPA derivatization.** Extracts for the analysis of the conversion of hydroxyectoine to ectoine (section 4.5.2) were analyzed by HPLC for both the ectoine content and the content of OPA amino reactive compounds at BAM following Schwibbert et al. (2011).
4.6.6  $^1$H and $^{13}$C nuclear magnetic resonance (NMR)

Further insight into the atomic structures of molecules in samples (e.g. small polar metabolites extracted from 0.25 L cultures of strains of *H. elongata ΔeutBΔeutCectB::ΩΔdoeD*) was obtained by recording $^1$H and $^{13}$C NMR spectra of samples dissolved in deuterium oxide. NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer operating at $^1$H frequencies of 300.13 MHz and carbon frequencies of 75.47 MHz, using as chemical shift references the residual water peak and acetonitrile as internal standard ($H_2O$: $\delta_H$ 4.79; CH$_3$CN: $\delta_C$ 119.68, 1.47 and $\delta_H$ 2.06) (Gottlieb et al., 1997).

4.7 Protein cloning techniques

4.7.1 Protein extraction

*For normalization of data.* Stored cells from culture aliquots (1 mL) were dried (65°C, overnight) and resuspended in demineralized water (1 mL). Cell lysis was promoted by incubation (80°C, 20 min) followed by shaking (RT, 30 min). The biomass debris was then sedimented by centrifugation (4°C, 13000 g, 30 min) and the total protein content in the supernatants was determined using the BCA test (section 4.7.2).

*For analysis in SDS-PAGE.* Bacterial cell pellets from the main cultures (10 mL each) were separately thawed, and resuspended in pre-chilled (<4°C) lysis buffer (50 mM NaH$_2$PO$_4$, 500 mM NaCl, 10 mM C$_3$H$_4$N$_2$; 6 mL each). Cells were disrupted by subjecting to hydrodynamic shearing through exposure of cell suspensions to sonic pulses while in a water/ice bath (30 min, 0°C). Cell debris was sedimented by centrifugation (4°C, 13000 g, 30 min) and the protein content of the supernatants was analyzed by the BCA test (section 4.7.2).
4.7.2 Protein Quantification

**BCA test.** The protein content in extracts was estimated by comparing the colorimetric detection registered for the product (blue purple, $\lambda_{\text{max}}$ 562 nm) of Cu$^+\ (4)\) that reacted with bicinehonicin acid (BCA) during BCA based tests (BCA Protein Assay Kit: Uptima, Montluçon, France) against photon absorptions registered for bovine serum albumin standards (BSA; 50, 100, 200, 300, 400 and 500 $\mu$g/mL). BCA blank, standards and samples (diluted if necessary) were pipetted (20 $\mu$L each) into different wells in 96 well microtiter plates, followed by addition of the BCA reagent (200 $\mu$L) and incubation (60 °C, 30 min). Absorbance of the samples was then measured (550 nm).

4.7.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

“Polyacrylamide gels are formed from the polymerization of acrylamide monomers in the presence of smaller amounts of N,N'-methylene-bisacylamide (bis-acrylamide). Acrylamide monomer is polymerized in a head to tail fashion into long chain and occasionally a bis-acrylamide molecule is built into the growing change, thus introducing a second site for chain extension that conclude in well define matrix of relatively well define structure. Polymerization is initiated by the addition of ammonium persulphate and the base N,N,N',N'-tetramethylenediamine (TEMED). TEMED catalyses the decomposition of persulphate ions to give free radicals." (Wilson and Walter, 2005)

**SDS-PAGE preparation.** Components for a 13 % polyacrylamide gel mixture [20 % acrylamide solution$^5$ (6.5 mL), separation gel buffer (1.5 M Tris-HCl, pH 8.8; 2.5 mL), 10 % SDS (100 $\mu$L), 10 % APS (50 $\mu$L) and TEMED (10 $\mu$L, last additive)] were combined in a beaker. The separating gel mixtures were poured into the gel cassettes (about two thirds of the total length of the gel phase) and covered with water to ensure a smooth surface gel polymerization (>1 h incubation at RT). The overlaying water was poured off from the separating gels and their surfaces were carefully dried with paper. The stacking gel (4 %

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$^4$ Cu$^+$ is formed from Cu$^{2+}$ cations after experimenting reduction due to reactions with peptide bonds

$^5$ 30 % acrylamide (32.7 mL) + 2 % bis-acrylamide (10 mL) + H$_2$O (7.3 mL)
acrylamide) still in solution [20 % acrylamide mixture (1 mL), stacking gel buffer (0.5 M Tris-HCl, pH 6.8; 2.5 mL), 10 % SDS (50 µL), 10 % APS (50 µL) and TEMED (10 µL)] was added into the gel cassette until the solution reached the cutaway edge of the gel plate. A comb was placed into this solution and left to set (>1 h). After the gels had set, the combs and the sealing belts of the gel cassettes were removed, the bottom reservoirs were filled with electrophoresis buffer (50 mM Tris, 370 mM glycerol, 0.1 % (w/v) SDS, pH 8.3-8.6) and the cassettes were assembled in the electrophoresis chamber. The top and bottom reservoirs were filled with electrophoresis buffer and the samples to be analyzed were loaded onto the gel as described below.

**Samples preparation and gel running conditions.** Diluted protein samples were mixed with 4xRSB buffer [0.05 M Tris-HCl, pH 6.8 (4.5 mL) + glycerol (5 mL, 50 % v/v), SDS (0.5 g, 5 % w/v), DTT (0.386 g, 4 % w/v), bromophenol blue (<10 mg) and H₂O de (to 10 mL)]. In the presence of DTT (which reduces disulfide bridges) and SDS (which binds strongly to the protein and provides a net negative charge), native structures of proteins in the samples were denatured by heating (95 °C, 10 min). Denatured protein samples (10 µl each) were loaded and the gel was run at a constant voltage (100 V) until bromophenol blue reached the bottom of the separating gel.

**Visualization of proteins separated in polyacrylamide gels.** The gel was placed in a fixation/staining solution (0.25 % w/v Coomassie Brilliant blue R-250 in a solution 5:4 methanol:acetic acid) in a plastic dish that was incubated on a slowly rotating platform (30 min). The fixative was removed and the gel washed (2-3 times) with destaining solution (5:4 methanol:acetic acid).

### 4.7.4 Recombinant protein production

Overexpression of recombinant proteins was performed using the pET Systems. The pET System uses pET plasmids with the bacteriophage T7 promoter, which is not recognized by *E. coli* RNA polymerase. These are therefore transformed into a host bearing the T7 RNA polymerase gene (λDE3 lysogen) for expression of target proteins.
The most widely used \( \lambda DE3 \) lysogens \( E. \ coli \) host is the BL21, which has the advantage of being deficient in both \( lon \) and \( ompT \) proteases. In \( \lambda DE3 \) lysogens the T7 RNA polymerase gene is under the control of the \( lacUV5 \) promoter, which allows some degree of transcription in the uninduced state. In the absence of further controls (as was the case of the experiments reported herein), this promoter is suitable for expression of many genes whose products have innocuous effects on host cell growth (Novagen, pET system manual). In this study, the plasmid pET-22b(+) and the host \( E. \ coli \) BL21 were used.

**4.7.4.1 \( E. \ coli \) in vivo test of activity of recombinant proteins**

Competent cells of \( E. \ coli \) BL21 were separately transformed with empty pET-22b(+), pET-22b\_doeA and pET-22b\_eutBC and selectively cultivated (37 °C, 180 rpm, overnight) on LB-1 amp100 agar plates.

**4.7.4.1.1 Analysis of gene toxicity and protein expression**

Transformed \( E. \ coli \) BL21 carrying pET-22b\_doeA and pET-22b\_eutBC were cultivated overnight (37 °C, 200 rpm) on LB-1 amp100 medium (20 mL, in a 100 mL capacity Erlenmeyer flasks). These overnight cultures (3 mL, each) were used to inoculate MM63-3 amp100 medium supplemented with 2 mM ectoine (100 mL each, in a 250 mL nose flasks). Cultures in minimal media were incubated (37 °C, 180 rpm) and samples (10 mL each) were taken just prior to induction (\( OD_{600nm} \approx 0.5 \)). Each of the cultures was then separated in two (50 mL cultures each). IPTG was added to one of each pair of cultures to reach a final concentration 1 mM; the other cultures were used as uninduced controls. Cell samples (each from 10 mL culture) were harvested (section 4.6) every hour after induction, proteins extracted for analysis in SDS-PAGE (section 4.7.1) and the protein content in the supernatants was estimated according to the BCA test (section 4.7.2).

**4.7.4.1.2 In vivo assay of recombinant proteins**

Cell aliquots of \( E. \ coli \) BL21 were separately transformed (section 4.4.4) with empty plasmid pET-22b(+), pET-22b\_doeA or pET-22b\_eutBC and selectively cultivated (37
°C, 180 rpm, overnight) on LB-1 amp100 medium (30 mL, in a 100 mL capacity Erlenmeyer flasks). These overnight cultures (5 mL, each) were used to inoculate MM63-3 amp100 medium supplemented with 3 mM ectoines (100 mL each, in a 250 mL nose flasks). Cultures in minimal media were incubated (30 °C, 180 rpm) and samples (15 mL) were taken just prior to induction (OD$_{600}$≈0.6) with IPTG (final concentration: 1 mM), as well as 2 and 4 h after induction, to analyze the intracellular concentration of ectoines. Cells in sampled cultures were harvested, extracted and analyzed according to sections 4.6 and 4.7.

4.8 Additional specialized computer analysis and databases

Protein and translated nucleotide databases were screened to find proteins of potential activity in the metabolism of ectoines using the NCBI BLAST tool and Halolox. Digital DNA sequences were administrated with Clone Manager and Primer Designer for vector construction purpose. Multiple sequence alignments of DNA sequences were constructed on a Sony Vaio computer using the ApE software. Alignments were copied in MS-Word and artificially modified with no alteration of the sequences in order to optimize the organization on the written report. Details for accessing these electronic sources are listed below.

- BLAST (Basic Local Alignment Search Tool): http://blast.ncbi.nlm.nih.gov/Blast.cgi
- Halolox: www.halolox.de
- Clone-Manager 7:
  Version 7.03, Scientific & Educational Software, Durham, USA
- Primer Designer:
  Version 3.0, Scientific & Educational Software, Durham, USA
- ApE, A plasmid editor v2.0.37
- Brenda: www.brenda.de

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4.9 Chemicals, purity and source

Enzyme reactions with purchased enzymes were performed with the buffers and reagents provided by the enzymes’ manufacturer (Fermentas, unless specified otherwise in the corresponding section). DNA and protein markers, as well as additional electrophoresis accessories (e.g., loading buffers and enzyme buffers) used in this work were purchased from Fermentas.

Acetic acid, 100 % glacial (Merck)  
Acetone, 99.8 % (ROTH)  
Acetonitrile, HPLC grade, (VWR)  
Agarose, Genetic techn. Qual. (ROTH)  
1-Aminoadamantan, 99 % (Merck)  
Ampicillin, 99 % (ROTH)  
Ammonium sulfate, ≥99 % (Merck)  
Ammonium persulfate (APS), ≥98 % (BioRad)  
Ammonium chloride, pure (Merck)  
Antibiotic broth medium No. 3 (Oxoid LTD)  
Acrylamide (Merck)  
Bis-acrylamide (Merck)  
Boric acid, 99 % (Fluka)  
Bovine serum albumin, BSA (Uptima)  
Bromophenol blue (Merck)  
Calcium chloride, 99 % CaCl2·2H2O (Merck)  
Casein peptone (ROTH)  
Coomassie Brilliant blue R-250 (ROTH)  
Chloroform, 99.8 % (Merck)  
Deuteriumoxid, >99.9 % (Merck)..  
DMSO, 99.5 % (Fluka)  
Diaminobutyric acid (L-2,4) (Sigma)  
Dithiotreitol (DTT), >99 % (Sigma)  
Ectoine, 96 % (Bitop)  
EDTA (disodium salt) (Serva)  
Ethanol, 99.8 % (ROTH)  
Ethidium bromide (Fluka)  
9-Fluorenlyl-methylchloroformate (FMOC-chloride), 97 % (Sigma-Aldrich)  
Glucose (monohydrated), D-C6H12O6·H2O (Merck)  
Glycerol, 99.5 % (ROTH)  
Gel red nucleic acid stain (Biotium)  
Hydrochloric acid, 37 % HCl (ROTH)  
Hydroxyectoine, 99 % (Bitop)  
Isopropanol, > 99.95 % (ROTH)  
Isopropyl-1-thio-β-D-galactopyranosid (IPTG) (Fermentas)  
Iron sulfate, 99.5 % FeSO4·7H2O (Merck)  
Kanamycin sulfate (Fluka)  
Norvaline (D/L), ~99 % (Fluka)  
Magnesium chloride, >98 % MgCl2·6H2O (Merck)  
Magnesium sulfate, 99 % MgSO4·7H2O (Merck)  
Methanol, Lichrosolv (ROTH)  
Potassium acetate, 99-100 % (Merck)  
Potassium hydrogen phosphate, >99 % K2HPO4 (ROTH)  
Potassium dihydrogen phosphate, >99 % KH2PO4 (ROTH)  
Potassium chloride KCl (Merck)  
Potassium hydroxide, KOH (ROTH)  
Select Agar, molecular genetics applications (Invitrogen)  
Sodium acetate, 99.5 % CH3COONa·3H2O (Sigma)  
Sodium Chloride, (Fluka)  
Sodium(dihydrogen)phosphate, NaH2PO4·H2O (Merck)  
Sodium dodecyl sulfate (SDS), recrystallized twice, C12H25O4SNa (SERVA).  
Sodium hydroxide (Merck)  
Sucrose, 99.5 % (ROTH)  
TEMED (N,N,N’,N’-tetramethylethylenediamine), 99 % (ROTH)  
Tetrahydrofuran (THF), 99.5 % (Merck)  
Tris, 99.9 % (ROTH)  
Tri-sodium citrate (dihydrated), 99 % (Merck)  
Yeast Extract, for bacteriology (ROTH)
5 Results

5.1 EctA independent ectoine biosynthesis

Grammann et al. (2002) reported the elimination of the ability of *H. elongata* to synthesize ectoine following the deletion of the *ectA* gene. The Δ*ectA* mutant has been shown to have limited growth ability on media containing above 5 % NaCl (Witt 2005). This mutant was used to define the hydroxyectoine conversion to ectoine situation (Figure 3). When grown at 11 % salinity (27 °C), *H. elongata* WT accumulated ectoine as a major compatible solute with less than 2 % (molar ratio) hydroxyectoine as part of the ectoines pool. It was also verified that under such conditions (i.e., in the absence of compatible solute supplementation) the Δ*ectA* mutant (which is unable to biosynthesize ectoine from glucose) was unable to grow. However, when 5 mM HE was provided in the medium growth was observed, although cultures did not reach the same optical density as for the WT. When cultured in the minimal media, the WT strain could achieve an optical density above 7, by biosynthesizing ectoines from glucose to a concentration equivalent to 3.5 mM in the medium. The amount of HE provided in the medium (5 mM) to compensate for the growth deficiency in the Δ*ectA* mutant was not sufficient, since growth stopped at an optical density of ~5 with an ectoine accumulation of only 1.5 mM. Analysis of the intracellular abundance of the ectoine pool revealed that during the growth phase in the Δ*ectA* mutant HE is accumulated and accounts for over 75 % (molar ratio) of the ectoine pool. Nevertheless, the relative abundance of HE reduced drastically as growth became stationary and ectoine became by far (>80 %) the dominant compatible solute found in the cytoplasm. The key finding of this experiment is that it clearly shows that when pure hydroxyectoine (>99 % w/w) was provided in the medium, ectoine was formed by an unknown pathway in the cytoplasm.
Results

Figure 3. Hydroxyectoine conversion to ectoine in *H. elongata* KB1 (Δ*ectA*) as it grows in minimal medium (Bp-11) at 27 °C. Legend is depicted in the figure. The wild type strain (green) can grow without compatible solute supplementation (unfilled symbols). The Δ*ectA* mutant (orange) could not grow at 11 % salinity (unfilled symbols) unless hydroxyectoine was provided in the medium (filled symbols, 5 mM). E=ectoine, HE=hydroxyectoine.

5.2 Relations among the target genes

A literature review and analysis of data bases led to the development of several hypotheses that might explain the conversion of hydroxyectoine to ectoine. The main hypotheses are synthesized in a hypothetical model to describe the ectoines' metabolism (Figure 4). This model involves genes both for the biosynthesis (*ectABCR*) and the degradation (*doiABXCD*) pathways. The hypothetical model follows an analogy for catabolism of hydroxyectoine as it is already known for ectoine. The DoeA is already known to cleave the ectoine ring, so it is likely that it may be able to cleave the hydroxyectoine ring as well (conversions 8-9 or 8-10). A potential first step in the conversion and catabolic pathways could use the same set of enzymes that are involved in ectoine catabolism allowing the potential removal of the hydroxyl group from a not cyclic substructure (9-13).
Figure 4. The ectoines' hypothetical cycle in *Halomonas elongata*. Schematic illustration of what is known about the metabolic pathways of ectoine and hydroxyectoine (correlations among molecules 1-8) and the hypothetical steps that might explain both hydroxyectoine conversion to ectoine and its degradation (correlations involving hypothetical metabolites 9-19). Although several other relations and metabolites could yet be built-in, only those more likely, as mentioned in the text, were included.

- Solid arrows represent enzymatic steps supported by experimental evidences
- Dashed arrows represent hypothetical enzymatic steps.

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The degradation of hydroxyectoine unequivocally involves at least one enzymatic step in which at least one hydroxylated metabolite loses a hydroxyl group. However, none of the enzymes known to participate in the metabolism of ectoines, either in biosynthesis or degradation, are associated with dehydroxylation activity.

In the search for potential dehydroxylating enzymes, gene *eutB* seems like a good candidate. It is homologous to a gene that codes for a protein that, along with nine others, was ectoine-induced in *Sinorhizobium meliloti* (Jebbar et al., 2005). Its predicted role in the “ectoine utilization” pathway could be related to dehydroxylation activity because *eutB* presents a significant homology to threonine dehydratases (EC4.3.1.19). Since threonine shares structural similarities with hypothetical metabolites (Figure 4, blue substructures), this gene became a target for future research. Among the dehydratases, some highly specific enzymes have been reported that catalyze reactions like those proposed for the removal of the hydroxyl group from hypothetical intermediates 9-13 and 17 (Murakami et al., 2009). Several dehydratases have been shown to accept more than one substrate, for example threonine and serine (Bornaes et al. 1992). Gene *eutB* was found next to *eutC* in several organisms (e.g. *S. meliloti* and *C. salexigens*), a gene coding for an enzyme (EutC) of high amino acid sequence homology to ornitine cyclodeaminases (EC4.3.1.12). Ectoines’ structures share similarities with molecules that are recognized by cyclodeaminases (i.e. figure 4, green substructures), Therefore, these were also included in the hypothetical model as a potentially reversible enzyme in conversion 6-19 and 8-17 (Lee et al., 2010), and were included in this study as well. These genes (*eutB* and *eutC*) have already been studied as part of the ectoine metabolism in *H. elongata* but the results did not implicate them in the metabolism of ectoine (Schwibbert et al., 2011).

The ectoines’ hypothetical model (Figure 4) suggests that the hydroxyectoine catabolism may be associated to the intermediates in the ectoine metabolism through successive enzymatic steps of dehydration (dehydroxylation) and transamination that might take place at several intermediate levels. Although some possible transaminating enzymes were identified, this group of enzymes tends to be of broad specificity (Schwibbert et al., 2011). Hence, this type of reaction (14-3, 15-4, 16-5 and 18-19) could be catalyzed by more than one enzyme (including for example, amino acid transaminases).
5.3 Analysis of recombinant proteins

The genes of unknown function but with the potential to participate in the metabolism of hydroxyectoine (i.e. doeA, eutB and eutC) were cloned in E. coli in order to test the corresponding recombinant proteins in vivo for any activity that altered the structure of ectoines. The results are shown below.

5.3.1 Construction of expression vectors

DNA sequences of H. elongata DSM 2581^T coding for doeA (1228 bp) or eutBC (2034 bp) were PCR-amplified and restricted with Ndel and HindIII for ligation into conveniently digested pET-22b(+) vectors. An additional vector for overexpression of N-terminal his tagged DoeA was constructed by insertion of the appropriate gene containing sequence into the single restricted (Ndel) pET-15b.

The insertion of the DNA sequences into the vectors was confirmed by agarose gel electrophoresis analysis of the products resulting from restriction of pure plasmids. DNA bands of expected sizes (Table 7) could be observed (Figure 5). The DNA concentration in these solutions was determined (0.02 and 0.03 mg plasmid/μL for pET-22b_doeA and pET-22b_eutBC, respectively). The DNA solutions were then used for sequencing the DNA fragment that had been inserted in the constructed vectors. The determined vector DNA sequences proved to be equal to those of the regions of target genes in H. elongata wild type (Figures 51 and 52).

Figure 5. Agarose gel electrophoresis to control the DNA insertion into expression vectors and to quantify the DNA before submission to DNA sequencing. a) lanes 1 and 6: DNA mass ruler (3 and 6 μL, respectively), lanes 2 and 3: products of restriction digest of pET-22b_doeA with EcoRV (4 and 8 μL, respectively), bands at approximately 2193 and 4404 bp have a good match for expected sizes; lanes 4 and 5: products of restriction digest of pET-22b_eutBC with EcoRV (4 and 8 μL, respectively), bands at 2792 and 4606 have a good match for the expected sizes. b) Agarose electrophoresis profile of DNA mass ruler provided by the manufacturer and used for the estimation of the DNA concentration (given in the text).
5.3.2 *In vivo* test of activity of recombinant proteins in *E. coli*

The expression of recombinant proteins using the constructed vectors (section 5.3.1) was assayed while experimental conditions for testing their activity *in vivo* were being established (Figure 53). The SDS-PAGE analysis of cell samples collected at different cultivation times revealed an over-expression of proteins (Figure 54). The sizes of the over-expressed proteins, as approximated from their migration on the PAGE gel, do match the expected sizes (DoeA, 44920 Da; EutB, 34541 Da and EutC, 33985 Da). Moreover, the over-expressed bands could also be observed in samples without induction due to basal expression. The induction of expression effectively increased the cytoplasmic concentration of target proteins so that the recombinant proteins became the major proteins in the cytoplasm. However, because of the small difference in size between EutB and EutC, it is not possible to distinguish whether or not the protein band corresponds to one or two target proteins (EutB and EutC).

After having observed relatively good growth and apparently proper protein expression (see above and figures 53 and 54), the potential of the recombinant proteins DoeA, EutB and EutC to modify the structures of the ectoines was tested in the expression host *E. coli* BL21. For this, pET systems suitable for over-expression of the target proteins (section 5.3.2) were grown in media supplemented with ectoines (Figure 6). It was observed that, for a given strain, supplementation with hydroxyectoine promoted better growth than did ectoine (Figure 6, a Vs b). Furthermore, the host strain with an empty plasmid grew slightly better than the strains carrying *doeA* (blue Vs dark red data series). Conversely, the strain with an empty plasmid did not grow as well as the strain carrying *eutBC* (blue Vs green data series).
Results

Figure 6. Growth behavior of *E. coli* BL21 cells expressing target proteins while cultivated at 37 °C in MM63-3 medium. Supplemented with a) 3 mM ectoine (E) and b) with 3 mM hydroxyectoine (HE). Legend is depicted in the figures. Arrows point to the coordinates at induction time with IPTG.

To assess any possible alterations to the chemical structure of ectoines as a result of the activity of recombinant proteins, I measured the concentrations of ectoines 4 h after induction of samples in both the medium and the cytoplasm (figure 6). With this information, a mass balance was calculated based on the decrease of the concentration of ectoines registered in the medium during the growth period (the amount expected to have been taken up), and the amount actually measured in the cytoplasm. The intracellular amounts of ectoines were expressed in terms of concentrations in relation to the volumes of culture from which the corresponding cells were harvested (Figure 7). The strain with an empty plasmid (no degradation) was considered as a negative control, and the plasmid carrying doeA (known to cleave the ectoine ring: Schwibbert et al., 2011) was used as a positive control. Using this method of evaluation, an ideal negative control should give a mass balance of zero (taken up=intracellular) while positive values would mean synthesis and negative values degradation.
In the systems analyzed for ectoine degradation (Figure 7a), *E. coli* BL21 with an empty plasmid took up less ectoine (0.3 mM) than strains carrying *doeA* (2.9 mM) or *eutBC* (2.4 mM). The comparison of the intracellular concentrations of ectoine (0.7, 0.3 and 1.0 mM; empty, *doeA*+ and *eutBC*+ systems, respectively) proved to be lower in the system carrying *doeA* (0.3 mM) than it should have been in the case of no degradation (above 0.7 mM observed in the negative control), especially considering the optical density at which the cells were harvested (0.59, 0.54 and 0.82, empty, *doeA*+ and *eutBC*+ systems, respectively). Thus, a slightly positive balance (0.5 mM) was observed in the negative control and a markedly negative balance (-2.6 mM) in the positive control, while the target test system (carrying *eutBC*) had a moderately negative balance (-1.5 mM).

In the systems investigated for hydroxyectoine degradation (Figure 7b), *E. coli* BL21 with an empty plasmid took up less hydroxyectoine (1.5 mM) than strains carrying *doeA* (3.7 mM) and a comparable amount to those carrying *eutBC* (1.1 mM). The comparison of the intracellular concentrations of hydroxyectoine (2.1, 0.4 and 0.8 mM; empty, *doeA*+ and *eutBC*+ systems, respectively), as for ectoine, proved to be lower in the system carrying *doeA* (2.1 mM) than it should have been in the case of no degradation (above 2.4 mM observed in the negative control), especially considering the optical density at which the cells were harvested (0.55, 0.40 and 0.78, empty, *doeA*+ and *eutBC*+ systems, respectively). Thus, a slightly positive balance (0.4 mM) was observed in the negative control and a markedly negative balance (-3.3 mM) in the positive control, while the target test system (carrying *eutBC*) had a moderately negative balance (-1.5 mM).
carrying doeA (0.4 mM) than it should have been assuming no degradation (above 2.1 mM observed in the negative control), especially considering the optical density at which the cells were harvested (0.78, 0.78 and 0.96; empty, doeA\(^+\) and eutBC\(^+\) systems, respectively). Thus, a slightly positive balance (0.6 mM) was observed in the negative control and a markedly negative balance (-3.3 mM) in the doeA\(^+\) system, while the subject system eutBC showed only a slightly negative balance (-0.3 mM).

Samples from the expression systems tested for degradation activity of ectoines were also analyzed by HPLC after FMOC derivatization (Figure 8). Signals that could be attributed to activity of the recombinant proteins were only observed in the systems carrying doeA. After comparison with standards (Figure 55 and 56), the unusual substances detected in system carrying doeA supplemented with ectoine were designated as \(\gamma\)-NADA (5, \(t_R=\) 9.2 min), \(\alpha\)-NADA (7, \(t_R=\) 10.1 min) and DA (4, \(t_R=\) 37.5 min). The pattern of signals registered when ectoine was degraded in the presence of DoeA is similar to that observed in the same system containing hydroxyectoine. Therefore, by analogy, it was presumed that the substances detected in the previous expression system when supplemented with hydroxyectoine should include the hydroxylated derivatives \(\gamma\)-NA-HO-DA (9), \(\alpha\)-NA-HO-DA (10) and HO-DA (11). The presence of 9 and 10 could not be confirmed based on a standard of chemically hydrolyzed hydroxyectoine because this reaction leads to the formation of at least four products (based on NMR analysis, figure 57) with similar retention times that match the molecules eluting in peak at \(\sim\)6 min (Figure 58). A mixture of products of the chemical hydrolysis of hydroxyectoine was subjected to enzymatic deacetylation. The products of this reaction were proved by NMR to contain 11 (Figure 37). This mixture was compared with the extract from \textit{E. coli} containing unknown metabolites derived from recombinant DoeA activity on hydroxyectoine, and with an extract of \textit{H. elongata} K2.3. This comparison confirmed that the metabolite eluting at \(\sim\)33 min is 2,4-diamino-3-hydroxybutyric acid, 11 (Figure 36). The degradation products associated with DoeA activity in the cytoplasm could also be detected in the medium from the corresponding \textit{E. coli} cultures, suggesting their excretion to the medium (Figures 55d and 58c).
Figure 8. HPLC profiles after FMOC derivatization of compounds extracted from E. coli BL21 cells harvested 4 h after induction during an activity test of target proteins. Samples correspond to growth curves in figure 5. For degradation products of a) Ectoine and b) Hydroxyectoine. Legend is depicted in the figures. Arrows point to signals of degradation products (also excreted to the medium, see figures 55 and 58). DA (4), γ-NADA (5), α-NADA (7), γ-NA-HO-DA (9), α-NA-HO-DA (10) and HO-DA (11)

5.4 Construction and analysis of deletion mutants

The function of genes potentially involved in the metabolism of ectoines was investigated through the creation of gene-defective mutants. These were analyzed by cultivation under conditions designed to challenge the mutants to proceed with the metabolism of ectoines. During cultivation, samples were harvested to observe the relations among the accumulated intermediates.
5.4.1 Construction of deletion mutants

5.4.1.1 Vector construction

*H. elongata* DNA sequences upstream (508 bp) and downstream (532 bp) of *doeA* or upstream (719 bp) and downstream (760 bp) of *eutB* were amplified by PCR and joined together by SOE-PCR (1017 and 1454 bp, respectively). A DNA fragment constructed for deletion of *eutC* (2261 bp) was obtained from Heidrich, G. (BAM, Berlin). After restriction with *NdeI* and/or *HindIII*, the sequences were ligated into pK18*mobsacB* vectors.

The construction of the vectors for deletion was confirmed by agarose gel electrophoresis analysis of products resulting from the restriction of pure plasmids. DNA bands of expected sizes (Table 7) were observed (Figure 9). The DNA concentration in these solutions was estimated (0.05, 0.05 and 0.025 μg of plasmid/μL for pK18*mobsacB* carrying Δ*doeA*, Δ*eutB* and Δ*eutC*, respectively). The DNA solutions were further used to determine the DNA sequence of the plasmid regions containing the inserted DNA (Figures 59 and 60). The DNA sequences were of high fidelity in the case of Δ*doeA* and Δ*eutC*, but a base mutation was found in the plasmid pK18*mobsacB_ΔeutB* (Figure 59b).

![Figure 9](image-url)  
Figure 9. Agarose gel electrophoresis profiles of digestion products of deletion vectors. a) 1 and 8: DNA mass ruler (3 and 6 μL, respectively); 2 and 3: products of restriction of pK18*mobsacB_ΔdoeA* with *EcoRV* and *NcoI* (4 and 8 μL, respectively); 4 and 5: products of restriction of pK18*mobsacB_ΔeutB* with *Alw44I* (4 and 8 μL, respectively); 6 and 7: products of restriction of pK18*mobsacB_ΔeutC* with *SmaI* (4 and 8 μL, respectively). b) Restriction products of pK18*mobsacB_ΔeutB* with *Alw44I* when digesting 50% less plasmid DNA than that used for gel a4-5; lanes 9, 10 and 11 (2, 4 and 8 μL, respectively). c) Agarose electrophoresis profile of DNA mass ruler provided by the manufacturer and used for the estimation of the DNA concentration (given in the text).
5.4.1.2 Gene deletions

The procedure for double crossover involving both flanking regions with vectors pK18mobsacB_ΔdoeA (6672 bp) and pK18mobsacB_ΔeutB (7122 bp) was not successful. Therefore, all ΔdoeA mutations included in this work were made with the plasmid pK18mobsacB::ΔdoeA constructed by Schwibbert et al. (2011). The ΔeutB mutation was maintained from strain KB49 constructed by Schwibbert et al. (2011).

5.4.1.2.1 Deletions with pK18mobsacB_ΔectC (6266 bp)

Plasmid pK18mobsacB_ΔectC was transferred into H. elongata strains KB43 (ΔdoeA) and KB49 (ΔeutB) as described in section 4.4.7. After the second homologous recombination step, colonies were screened for the absence of ectC using in situ PCR. The amplification of two fragments of different sizes (expected sizes: 496 and 910 bp) from different colonies was the first indication that the deletions were successful (Figure 10). The shorter fragments matched the size of the fragment amplified from the positive control, the pK18mobsacB_ΔectC reasonably well. However, the fragment was slightly larger (>500 bp) than it should have been (496 bp). Therefore, selected colonies were analyzed further by loading less DNA on the gels. At the same time, a section of the ectA gene was amplified in order to confirm that the strains were actually H. elongata and not E. coli (Figure 10c). Hence, the isolated strains (1 and 22) were designated as H. elongata KB43.1 (ΔdoeAΔectC) and KB49.1 (ΔeutBΔectC), respectively.

![Figure 10. Agarose gel electrophoresis profile of colonies in situ PCR screened for ΔectC mutation. a) In colonies derived from KB43 (lanes 1-13) b) In colonies derived from KB49 (lanes 21-25) c) Amplification of a DNA fragments from colonies used for lanes a1 and b22 including either ΔectC or ectC (left side of the marker) and a section of the ectA gene (right side of the marker). M1=Marker (1 Kb). M2=Marker (100 b), P= pK18mobsacB_ΔectC, WT= genomic DNA isolated from H. elongata wild type.](image-url)
5.4.1.2.2 Deletions with pK18mobsacB::ΔdoeA (~7722)

The plasmid pK18mobsacB::ΔdoeA was transferred into *H. elongata* strains KB49 (ΔeutB), KB49.1 (ΔeutBΔectC) and KB1 (ΔectA) as described in section 4.4.7. After the second homologous recombination step, colonies were screened for the absence of doeA using *in situ* PCR. The second homologous recombination step was repeated every time that no positive colonies were detected in groups of less than 10 colonies. The amplification of fragments of different sizes (expected sizes: 305 and 1502 bp) from different colonies was the first indication that the deletions were successful (Figure 11). The shorter fragment matched the size of the fragment amplified from the positive control for deletion (pK18mobsacB::ΔdoeA, ≈305 bp) reasonably well. The first two groups of colonies of ΔeutB screened for ΔdoeA mutation (lanes 1-3 and 11-18) did not yield any positive colonies. Positive colonies with ΔdoeA could only be detected (in lanes 34 and 35) after a third repetition of the second homologous recombination, in the ΔeutB strain (*H. elongata* KB49.2, ΔeutBΔdoeA). For the detection of the ΔdoeA mutation in the ΔeutBΔectC mutant, only three colonies were screened, one of which (in lane 4) was positively identified as *H. elongata* KB49.1.1 (ΔeutBΔectCΔdoeA). Of the five colonies of the ΔectA strain that were screened for the doeA deletion (lanes 19-23), four (lanes 19, 20, 22 and 23) were positive (*H. elongata* KB1.12, ΔectAΔdoeA).

![Figure 11. Agarose gel electrophoresis profile of colonies in situ PCR screened for ΔdoeA mutation. a) In colonies derived from strains KB49 (lanes 1-3) and KB49.1 (lanes 4-6) b) In colonies derived from strains KB49 (lanes 11-18) and KB1 (lanes 19-23) c) In colonies derived from KB49 (lanes 31-35) and from cells ΔdoeA targeted for deletion of eutC (A). P= pK18mobsacB::ΔdoeA. H= *H. elongata* ΔeutB::pK18mobsacB::ΔdoeA. M=Marker (1 kb).](#)
5.4.1.2.3  Deletions with pK18mobsacB::ΔeutC (7963 bp)

The plasmid pK18mobsacB::ΔeutC was transferred into *H. elongata* strains DSM 2581<sup>T</sup> (WT) and KB43 (ΔdoeA), as described in section 4.4.7. After the second homologous recombination step, colonies were screened for the absence of *eutC* using *in situ* PCR. Screening was first performed with primers used for the synthesis of the flanking regions (He366′0F1 and He365′8R1), but amplification was only successful with the plasmid and not on *Halomonas* strains (data not shown). The problem was solved with new primers specially designed for screening (He366′0F2 and He365′8R2). The amplification of fragments of different sizes (expected sizes: 676 and 1619 bp) from different colonies was the first indication that the deletion was successful (Figure 12). It was also observed that, in most of the colonies, the amplified sequences matched the size of the section amplified from the positive control for deletion, the pK18mobsacB::ΔeutC (≈676 bp) reasonably well. Of the four colonies of the KB43 strain screened for ΔeutC mutation (Figure 12a, lanes 1-4), three were clearly positive (lanes 2-4) and were designated as *H. elongata* KB43.2 (ΔdoeAΔeutC). Screening of colonies grown from wild type cells (Figure 12a, lanes 5-12) led to the identification of five colonies lacking the *eutC* gene (lanes 6, 8, 9, 11 and 12) out of eight colonies screened. Cells belonging to these five colonies were defined as *H. elongata* KB50 (ΔeutC).

![Figure 12. Agarose gel electrophoresis profile of colonies in situ PCR screened for ΔeutC mutation. a) In colonies of *H. elongata* strains KB43 (ΔdoeA): lanes 1-4; and in the WT: lanes 5-12. P= pK18mobsacB::ΔeutC. M= Marker (1 kb). b) Agarose electrophoresis profile of 1 kb DNA ladder provided by the manufacturer.](image-url)
5.4.2 Hydroxyectoine conversion to ectoine

The ability of *H. elongata* strains DSM 2581<sup>T</sup> (WT), KB43 (∆doeA), KB42 (∆doeB), KB49 (∆eutB), KB50 (∆eutC), WUB01 (∆ectC), KB43.1 (∆doeA∆ectC), KB43.2 (∆doeA∆eutC), KB49.1 (∆eutB∆ectC), KB49.1.1 (∆eutB∆ectC∆doeA), KB49.2 (∆eutB∆doeA), KB1 (∆ectA) and KB1.12 (∆ectA∆doeA) to convert hydroxyectoine into ectoine was studied at the molecular level in the presence of glucose. For this study were used the same conditions that had been previously established to study conversion ability (section 5.1: 28 mM Glucose, 5 mM HE), but at 30 °C. It should be noted that these conditions were similar to those described in section 5.4.3 (with 6 % NaCl) during the first growth phase, with the exception of the salinity level (11 % NaCl).

It was observed (Figure 13) that strains with defective biosynthesis of ectoine from glucose had a reduced ability to grow at high salinity (11 %), even when compatible solutes were available in the medium. The most affected strains were those carrying the ∆ectA mutation (Figure 13, orange and red triangles). The lowest total accumulation of ectoines (~4 mmol/mg of protein) was registered in the *H. elongata* strains KB49.1 and KB49.1.1.

![Figure 13. Growth curves of different strains of *H. elongata* in Bp-11 medium (28 mM G) supplemented with 5 mM hydroxyectoine at 30 °C. The total content of intracellular ectoines accumulated during the growth is indicated as discontinued lines. Legend is depicted in the figure.](image)
To facilitate the analysis of the data, the results of experiments in figure 13 were grouped into two categories: strains with full ectoine biosynthesis capacity (Figure 14) and those affected in the biosynthesis pathway (Figure 15). In all of the strains studied, however, ectoine could be detected (Figure 14b and 15b), even in those incapable of de novo biosynthesis of ectoine from glucose.

Despite the great similarities among strains with full ectoine biosynthesis capacity (Figure 15), the highest level of ectoine were observed in wild type cells (green circles), and the lowest levels of ectoine were detected in single mutants \( \Delta \text{eutC} \) (light blue circles) and \( \Delta \text{doeB} \) (light green circles). The \( \Delta \text{eutC} \) single mutant cells, however, increased the level of ectoine while in the log phase. Similar to \( \Delta \text{doeB} \), the single mutant \( \Delta \text{doeA} \) cells (yellow green circles) maintained a lower level of ectoine than the other strains during the growth phase. On the other hand, the strains that were best at cleaving the hydroxyectoine ring (Figure 16b) were the single mutants \( \Delta \text{eutB} \) (yellow green triangles of black line) and \( \Delta \text{eutC} \) (light blue triangles), while \( \Delta \text{doeB} \) (light green triangles) and the double mutant \( \Delta \text{eutB}\Delta \text{doeA} \) (pink triangles) were the worst.

The deletion of the \( \text{ectC} \) gene in \( \text{H. elongata} \) strongly reduces the amount of ectoine accumulated (Figure 15b, green Vs blue circles). In addition, the amount of ectoine biosynthesized under these conditions did not correspond in magnitude to the amount of hydroxyectoine cleaved (blue circles Vs blue triangles). This effect is transiently stronger when \( \text{doeA} \) is deleted as a second mutation (dark read circles Vs dark read triangles). However, the ability to synthesize ectoine is recovered as the stationary phase approaches. A more effective inhibition in the amount of ectoine accumulated is achieved when the \( \Delta \text{ectC} \) mutation is accompanied by the \( \Delta \text{eutB} \) mutation, either as \( \Delta \text{eutB}\Delta \text{ectC} \) (dark yellow circles) or \( \Delta \text{eutB}\Delta \text{ectC}\Delta \text{doeA} \) (dark pink circles), which behave similarly. The lowest accumulation of ectoine was observed in strains with deleted \( \text{ectA} \) (\( \Delta \text{ectA} \) and \( \Delta \text{ectA}\Delta \text{doeA} \); orange and red circles respectively), which also showed the maximum intracellular concentration of hydroxyectoine. The intracellular amount of hydroxyectoine decreased in all of the strains with increasing cultivation time.
Figure 14. Evaluation of the hydroxyectoine-ectoine conversion ability in strains of *H. elongata* with mutations that do not affect the biosynthesis of ectoines from glucose and the WT. a) Growth curves in Bp-11 medium (28 mM G) supplemented with 5 mM hydroxyectoine (triangles) at 30 °C; the total content of intracellular ectoines accumulated as they grow is indicated with discontinued lines. b) Intracellular abundance of ectoines accumulated during growth of curves in figure 14a. Legend is depicted in the figures.
Figure 15. Evaluation of the hydroxyectoine-ectoine conversion ability in strains of *H. elongata* with defective genes for the biosynthesis of ectoine from glucose and the WT.  

a) Growth curves in Bp-11 medium (28 mM G) supplemented with 5 mM hydroxyectoine (triangles) at 30 °C; the total content of intracellular ectoines accumulated as they grow is indicated with discontinued lines. 

b) Intracellular abundance of ectoines accumulated during growth of curves in figure 15a. Legends are given on the chart.
Cell extracts from cells growing in cultures with an initial concentration of glucose of 28 mM and supplemented with 5 mM HE (section 4.5.2 and figure 13) were also analyzed for potential intermediates (amino reactive with OPA) accumulated during the conversion of hydroxyectoine to ectoine. In general, the composition of samples was similarly complex to that illustrated in the HPLC profile of OPA modified compounds extracted from *H. elongata* WT (Figure 16). Nevertheless, the relative abundance of some signals had variations, being remarkable in the strains carrying the ΔdoeB (Figure 17), ΔectC (Figure 18) and ΔectA (Figure 19) mutations.

OPA amino reactive metabolites extracted from WT cells included glutamate (Glu), γ-NADA (5) and α-NADA (7) as the major metabolites (Figure 16). Glutamate was the most abundant, followed by γ-NADA and then α-NADA. Over 10 unidentified substances could be detected as traces. The deletion of doeB increased the relative amount of α-NADA to a level greater than that of γ-NADA (Figure 17). Conversely, the deletion of ectC increased the accumulated γ-NADA (Figure 18) tremendously. On the other hand, deletion of ectA leveled the accumulated amounts of γ-NADA and α-NADA (Figure 16 Vs 19), apparently because of a reduction in the abundance of γ-NADA.

Since OPA derivatization is not suitable for a simple detection of DA, random samples were analyzed by HPLC after FMOC derivatization, but only traces of DA could be detected (data not shown).
Figure 16. HPLC profiles of OPA amino reactive metabolites extracted from cells of *H. elongata* WT harvested from cultures grown in Bp-11 medium (28 mM G) supplemented with 5 mM hydroxyectoine incubated at 30 °C. Three samples harvested at different cultivation time were analyzed and chromatograms are shown overlapped. Comparison of major signals in this profile with amino acid standards and ectoine hydrolysis products show good match for glutamate (≈ 3.3 min, Glu), γ-NADA (≈ 8.2 min, 5) and α-NADA (≈ 8.9 min, 7). Similar profiles were observed for single mutants ΔdoeA, ΔeutB and ΔeutC.

Figure 17. HPLC profile of OPA amino reactive metabolites extracted from cells of *H. elongata* KB42 (ΔdoeB) harvested from cultures grown in Bp-11 medium (28 mM G) supplemented with 5 mM hydroxyectoine incubated at 30 °C. Three samples harvested at different cultivation time were analyzed and chromatograms are shown overlapped. Comparison of major signals in this profile with amino acid standards and ectoine hydrolysis products show good match for glutamate (≈ 3.3 min, Glu), γ-NADA (≈ 8.0 min, 5) and α-NADA (≈ 8.6 min, 7).
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Figure 18. HPLC profile of OPA amino reactive metabolites extracted from cells of *H. elongata* KB43.1 ($\Delta$doeA$\Delta$ectC) harvested from cultures grown in Bp-11 medium (28 mM G) supplemented with 5 mM hydroxyectoine incubated at 30 °C. Three samples harvested at different cultivation time were analyzed and chromatograms are shown overlapped. Similar profiles with high abundance of $\gamma$-NADA (5) were observed for *H. elongata* strains $\Delta$ectC, $\Delta$eutB$\Delta$ectC and $\Delta$eutB$\Delta$ectC$\Delta$doeA. Glu= glutamate

Figure 19. HPLC profile of OPA amino reactive metabolites extracted from cells of *H. elongata* KB1 ($\Delta$ectA) harvested from cultures grown in Bp-11 medium supplemented with 5 mM hydroxyectoine incubated at 30 °C. Three samples harvested at different cultivation time were analyzed and chromatograms are shown overlapped. Comparison of major signals in this profile with amino acid standards and ectoine hydrolysis products showed good match for glutamate ($\approx$ 3.3 min, Glu), $\gamma$-NADA ($\approx$ 8.4 min, 5) and $\alpha$-NADA ($\approx$ 9.2 min, 7). Similar profile was observed for *H. elongata* KB1.12 ($\Delta$ectA$\Delta$doeA).
5.4.3 Ectoines as a carbon source

5.4.3.1 H. elongata DSM 2581<sup>T</sup>

*H. elongata* DSM 2581<sup>T</sup> (WT) was used as a reference for normal growth when glucose and ectoines were offered simultaneously as a carbon source (Figure 20). The growth showed diauxic behavior, with a lag phase separating the two growth phases which occurred at different optical densities depending on the carbon sources available. The lag phase occurred at the highest cell density when ectoine was offered in the medium (OD ≈ 0.35). When hydroxyectoine was offered in the medium, the lag phase occurred at OD = 0.21. Both lag phases occurred at higher cell density than the maximum optical density reached by the wild type strain growing in the same amount of glucose (1 mM) as sole carbon source (OD = 0.14). It was also noted that the normal behavior included a significantly shorter lag phase when the carbon source was switched from glucose to ectoine than when it was changed from glucose to hydroxyectoine.

![Figure 20. Growth curves of *H. elongata* WT (DSM 2581<sup>T</sup>) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to $\mu_{\text{max}}$ when using ectoines as energy are indicated in the growth curves as black symbols.](image-url)
The maximum growth rate achieved by the wild type cells before glucose was consumed (first growth phase) in media containing ectoine (0.31 h⁻¹) and hydroxyectoine (0.24 h⁻¹) was faster than the maximum growth rate on only ectoines as carbon source, second growth phase (0.23 and 0.12 h⁻¹, for growth on E and HE, respectively) (table 9, page 92). In addition, the maximum growth rate achieved when ectoine was used as a carbon source (0.23 h⁻¹) was almost two folds greater than the maximum growth rate registered when utilizing hydroxyectoine (0.12 h⁻¹).

5.4.3.2 *H. elongata* KB49 (ΔeutB)

The deletion of eutB in *H. elongata* DSM 2581ᵀ did not change the general growth behavior observed for the wild type strain when the two carbon sources, glucose and ectoines, were offered simultaneously (Figure 20 Vs 21, circles). However, *H. elongata* KB49 (ΔeutB) needed longer than the WT to achieve the maximum growth rate on hydroxyectoine as a carbon source (Figure 20 Vs 21, triangles).

![Figure 21. Growth curves of *H. elongata* KB49 (ΔeutB) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E= glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to μ_max when using ectoines as energy are indicated in the growth curves as black symbols.](image-url)
5.4.3.3 *H. elongata* WUB01 (Δ*ectC*)

The deletion of *ectC* in *H. elongata* DSM 2581^T^ decreased the optical density at which the lag phase for switching the carbon source from glucose to ectoine was observed, from 0.35 (in the WT) to 0.23 (in Δ*ectC*); figure 20 Vs 22, circles, respectively. This effect was not observed, however, for the change from glucose to hydroxyectoine where the lag phase continued to occur at OD ~ 0.21. Nevertheless both *H. elongata* WUB01 (Δ*ectC*) and *H. elongata* KB49 (Δ*eutB*), needed longer than the wild type to achieve the maximum growth rate on hydroxyectoine as carbon source (Figure 20 Vs 22, triangles).

![Figure 22. Growth curves of *H. elongata* WUB01 (Δ*ectC*) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to μ_max when using ectoines as energy are indicated in the growth curves as black symbols.](image-url)
Cultivation of *H. elongata* WUB01 under conditions similar to those in figure 22 was performed on a larger scale (Figure 23). Screening for FMOC-modified compounds allowed the detection of DA (4) and γ-NADA (5) in cells growing in both ectoine and hydroxyectoine as a carbon source. The profiles were similar with the exception of a minor compound (tR ~ 33 min) detected in the culture growing on hydroxyectoine. This minor compound could be one (11) of those observed in the *in vivo* test of recombinant DoeA (Figures 8b and 36). Moreover, the accumulated 5 seemed to decrease during the growth phase on HE.

**Figure 23.** HPLC profile of FMOC reactive metabolites extracted from *H. elongata* WUB01 (ΔectC) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 4 = DA, 5 = γ-NADA, 11 = HO-DA

### 5.4.3.4 *H. elongata* KB50 (ΔeutC)

The deletion of *eutC* in *H. elongata* had little effect on the optical density at which the lag phases to switch the carbon source from glucose to ectoines occurred (Figure 20 Vs 24). However, deletion of *eutC* led to an enhancement in the maximum growth rate (from 0.23 to 0.27 h⁻¹) on ectoine as a carbon source (Figure 20 Vs 24, circles). Conversely, the absence of *eutC* reduced strains’ ability to use hydroxyectoine as a carbon source, as reflected by the long lag phase required to adjust the metabolism from the mode used during the first growth phase to that required to use hydroxyectoine as a sole carbon source. Once achieved, this adaptation resulted in a reduced maximum growth rate (from 0.12 to 0.09 h⁻¹, figure 20 Vs 24, triangles).
Cultivation of *H. elongata* KB50 under conditions similar to those in figure 24 was performed on a larger scale (Figure 25). The HPLC screening of compounds in cell extracts after FMOC derivatization allowed the growth phase dependent detection of DA (4) and γ-NADA (5) (or glutamate) in cells growing in ectoine. However, no 5 was accumulated when hydroxyectoine was available as a carbon source. Instead, a broad peak was observed at 15-20 min. Additional differences included a minor compound (tᵣ=33 min) detected in the culture growing on hydroxyectoine. As in the case of the single mutant WUB01 (ΔectC), this minor compound could be one (11) of those observed in the *in vivo* test of DoeA (Figure 8b, page 36). The culture having hydroxyectoine available could not grow on it in 67 hours of cultivation. While trying to grow on hydroxyectoine, compound 4 was the major FMOC reactive metabolite accumulated (Figure 25b). Whereas *H. elongata* KB50 (ΔeutC) reached the stationary phase on ectoine as a carbon source with the almost complete transformation of 4 and accumulating mainly 5, *H. elongata* WUB01 (ΔectC) became stationary accumulating both 4 and 5 (Figure 23a Vs 25a).
Results

Figure 25. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB50 (ΔeutC) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 5 = γ-NADA, 4 = DA, Glu = glutamate

5.4.3.5 *H. elongata* KB13 (ΔectB)

The maximum growth achieved by *H. elongata* KB13 in the presence of glucose in the glucose-ectoine medium (0.29, figure 27) was reduced in relation to the wild type (0.35, figure 20) and was slightly higher than the maximum first growth achieved in the system with the glucose-hydroxyectoine medium (~ 0.27).

Figure 26. Growth curves of *H. elongata* KB13 (ΔectB) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to $\mu_{\text{max}}$ when using ectoines as energy are indicated in the growth curves as black symbols.
5.4.3.6 *H. elongata* KB30 (ΔectR)

The deletion of *ectR* in *H. elongata* significantly increased the maximum growth rates achieved when glucose was still available (first growth phase), reaching values about 1.5 times those recorded for the wild type (Table 9). The maximum first growth achieved in the glucose-ectoine medium (~0.24, figure 27) was reduced in relation to the wild type (0.34, figure 20), and was very similar to the first maximum growth achieved on the glucose-hydroxyectoine medium (~0.25). In strain KB30 (ΔectR), however, the use of ectoine as a carbon source seemed to continue immediately after glucose depletion with no observable lag phase (Figure 27, circles). The distinction between the two growth phases could only be noticed from the differences in the growth rates.

![Growth curves of *H. elongata* KB30 (ΔectR) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to µmax when using ectoines as energy are indicated in the growth curves as black symbols.](image)

The maximum growth rate on ectoine as a carbon source (0.16) was significantly reduced in comparison with that observed in the WT (0.23). To change the metabolism adapted for first maximum growth rate (0.37 h⁻¹) to a mode enabling the second maximum growth rate (0.65 h⁻¹), the ΔectR strain needed longer than the WT. When
hydroxyectoine was the only carbon source, the maximum growth represented a reduction by half (0.65 h⁻¹) relative to the WT (Figure 20 Vs 27, triangles).

Cultivation of *H. elongata* KB30 under conditions similar to those in figure 27 was performed on a larger scale (Figure 28). The screening of FMOC derivatized compounds allowed the detection of DA (4), γ-NADA (5) and traces of a minor compound (t<sub>R</sub>~ 16 min) in cells growing in either ectoine or hydroxyectoine as a carbon source. Compound 4 was significantly accumulated mainly during the reduced growth phases (not clearly lag phases), but only traces were detected in the stationary phase where compound 5 became the most prominent FMOC reactive metabolite.

![Figure 28. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB30 (ΔectR) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 4 = DA, 5 = γ-NADA, Glu = glutamate](image)

5.4.3.7 *H. elongata* KB1 (ΔectA)

The deletion of ectA in *H. elongata* led to similar effects in both the behavior of the curves and in the magnitude of critical parameters as those observed in strain KB30 (ΔectR) when grown on glucose-ectoines media (Figure 27 Vs 29).

Cultivation of *H. elongata* KB1 (ΔectA) under conditions similar to those in figure 29 was performed on a larger scale (Figure 30). The screening of FMOC derivatized compounds allowed the detection of DA (4) and γ-NADA (5) (or glutamate) in cells growing in both ectoine and hydroxyectoine as a carbon source. The abundance of metabolite 5 was normally low becoming prominent only in the stationary phase of the culture grown on ectoine. Poor accumulation of 11 can be observed when hydroxyectoine was available in the medium.
Results

Figure 29. Growth curves of *H. elongata* KB1 (Δ*ectA*) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to $\mu_{\text{max}}$ when using ectoines as energy are indicated in the growth curves as black symbols.

Figure 30. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB1 (Δ*ectA*) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 5 = γ-NADA, 4 = DA, 11 = HO-DA, Glu = glutamate

While the strain KB1 (Δ*ectA*) accumulated 4 and 5 in the stationary phase of growth on glucose-ectoine medium (Figure 30a), the strain KB30 (Δ*ectR*) accumulated only 5 (Figure 28a). Additional differences between these two strains include the easier observation in Δ*ectA* than in Δ*ectR* of metabolite 11, and a broad peak eluting at 15-20 min (present also in Δ*eutC*: Figure 25).

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5.4.3.8 *H. elongata* KB49.1 (**ΔeutBΔectC**)

The deletion of *ectC* in the parental strain *H. elongata* KB49 (**ΔeutB**) resulted in a double deletion mutant *H. elongata* KB49.1 (**ΔeutBΔectC**). This mutant was dominated by the **ΔectC** phenotype, as observed for *H. elongata* WUB01 (**ΔectC**) when grown on a glucose-ectoine medium (Figure 22 Vs 31, circles). The growth on glucose-hydroxyectoine, however, seemed to be influenced by both mutations, **ΔeutB** and **ΔectC**, because the lag phase required to achieve growth on hydroxyectoine was longer and better defined than it was in either single mutant (Figure 21 and 22 Vs 31, triangles).

![Figure 31. Growth curves of *H. elongata* KB49.1 (**ΔeutBΔectC**) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to $\mu_{\text{max}}$ when using ectoines as energy are indicated in the growth curves as black symbols.](image)

Cultivation of *H. elongata* KB49.1 (**ΔeutBΔectC**) under conditions similar to those in figure 31 was performed on a larger scale (Figure 32). The screening of FMOC derivatized molecules allowed the detection of DA (4) and γ-NADA (5) in cells growing in both ectoine and hydroxyectoine as a carbon source. An additional minor compound
detected in cells growing on hydroxyectoine (t_R = 33 min) could be one (11) of those observed during the in vivo test of recombinant DoeA (Figures 8b and 36).

Figure 32. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB49.1 (ΔeutBΔectC) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 5 = γ-NADA, 4 = DA, 11 = HO-DA

5.4.3.9 *H. elongata* K2.3 (BAM 538, ΔeutBΔeutCectB::ΩΔdoeD)

The maximum growth achieved by *H. elongata* K2.3 at the end of the first growth phase in the glucose-ectoine medium (0.32, figure 33) was reduced relative to the wild type (0.35, figure 20), but similar to that observed in the glucose-hydroxyectoine medium (~0.30). Thus, the inactivation of genes *ectB*, *eutB*, *eutC* and *doeD* almost leveled the optical density at which the stationary phases for switching from glucose to ectoines as a carbon source occurred in *H. elongata* K2.3, in a way similar to that observed in *H. elongata* KB13 (ΔectB) (Figure 26 Vs 33). In fact, the overall growth of strain K2.3 in the glucose-ectoine medium seemed to be dominated by the ΔectB mutation but with a slight reduction in the maximum growth rates. Conversely, growth on the glucose-hydroxyectoine medium revealed that strain K2.3 had severe problems for using hydroxyectoine. During the phase where there should have been growth on hydroxyectoine as a carbon source, it is instead observed cell density oscillations, as if stationary and growth phases were periodically alternating with one another. Since the growth oscillations did not reach an optical density above that achieved during first maximum growth, no net growth could be concluded. However, growth was not monitored beyond 70 h, at which point net growth on hydroxyectoine could perhaps have been achieved.
Results

Figure 33. Growth curves of *H. elongata* K2.3 (ΔeutBΔeutCectB::ΩΔdoeD) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations. Growth rates were calculated from average curves in 1 h intervals and data points corresponding to $\mu_{\text{max}}$ when using ectoines as energy are indicated in the growth curves as black symbols.

Cultivation of *H. elongata* K2.3 under conditions similar to those in figure 33 was performed on a larger scale (Figure 34). The screening of FMOC derivatized molecules allowed the detection of DA (4) and $\gamma$-NADA (5) in cells growing in both ectoines. When grown on hydroxyectoine as a carbon source no 5 was significantly accumulated, and an additional major compound ($t_{\text{R}} \sim 33$ min) dominated the composition of the metabolite mixture. The analysis of the ectoine pool in these samples reveled that ectoine was present as the minor UV active component (Figure 35).

Jhonny Correa: Hydroxyectoine metabolism in *Halomonas elongata*
Cultivation of *H. elongata* K2.3 under conditions similar to those in figure 34 was up-scaled to 250 mL culture as outlined in section 4.6.3. The extracted metabolites contained the target unidentified metabolite ($t_R \sim 33$ min). By comparison with a standard prepared by enzymatic deacetylation of the compounds resulting of the chemical hydrolysis of HE using Acylase I from *Aspergillus melleu* (Fluka: 01818), the new metabolite was shown to be HO-DA (11). This was the same compound observed as a result of recombinant DoeA activity on hydroxyectoine in *E. coli* (Figures 36 and 37).
Figure 36. HPLC comparison of FMOC amino-reacted molecules to identify 2,4-diamino-3-hydroxybutyric acid (HO-DA) as a novel metabolite. a) Hydrolyzed hydroxyectoine: four products. b) Reaction mixture resulting of subjection of hydrolyzed hydroxyectoine to enzymatic deacetylation. c) Metabolites extracted from the cytoplasm of *E. coli* BL21 cells expressing DoeA while growing on HE supplemented media. Sample was taken 4 h after induction (Figure 6). d) Metabolites extracted from the cytoplasm of *H. elongata* K2.3 mutant cells (ΔeutBΔeutCectB::ΩΔdoeD) challenged to grow on HE under conditions indicated in section 4.6.3 to reproduce results in figure 34. Cells were harvested while trying to use HE as carbon source. $^{13}$C NMR profiles of samples a, b, and d are compared in Figure 37.

Jhony Correa: Hydroxyectoine metabolism in *Halomonas elongata*
Figure 37. Comparison of $^{13}$C NMR spectra to confirm the existence of 2,4-diamino-3-hydroxybutyric acid as a new metabolite. a) hydroxyectoine hydrolysis products (four products). b) molecular mixture resulting of the enzymatic deacetylation of a. c) cytoplasmic extract of strain K2.3. Arrows point to signals corresponding to novel metabolite synthesized in vitro (b) and as a novel metabolite (c). HPLC profiles of FMOC amino-reacted molecules in these samples are shown in figure 36.
5.4.3.10  *H. elongata KB43 (ΔdoeA)*

The deletion of *doeA* in *H. elongata* significantly reduced its ability to grow on ectoines as a carbon source (Figure 38). Despite this, a limited growth on ectoine was observed (Figure 38, circles). The reduction in growth seemed to be more notorious on hydroxyectoine, since it seemed to be abolished (Figure 38, triangles). Nevertheless, contrary what was observed on the ectoine medium, the corresponding growth curve did show relatively high variability during the stationary phase, as indicated by the error bars.

![Growth curves of *H. elongata KB43 (ΔdoeA)* when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations.](image-url)
5.4.3.11  *H. elongata KB43.2 (ΔdoeAΔeutC)*

The deletion of *eutC* in *H. elongata* KB43 (Δ*doeA*) resulted in a double mutant strain KB43.2 with slightly reduced growth ability on ectoines relative to the parent strain, Δ*doeA* (Figure 38 Vs 39). The growth curves looked similar regardless of whether ectoine or hydroxyectoine was provided as an additional carbon source in the medium.

![Growth curves of *H. elongata* KB43.2 (Δ*doeAΔeutC*) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations.](image)

Cultivation of *H. elongata* KB43.2 under conditions similar to those in figure 39 was performed on a larger scale (Figure 38). The screening of FMOC derivatized molecules allowed the detection of only low levels of glutamate (or 5). Glutamate remained at detectable levels in the cultures with ectoine, but was reduced to trace amounts in the extract from culture with hydroxyectoine.
Jhonny Correa: Hydroxyectoine metabolism in *Halomonas elongata*

Figure 40. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB43.2 (ΔdoeAΔeutC) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM).

5.4.3.12 *H. elongata* KB42 (ΔdoeB)

The deletion of doeB in *H. elongata* significantly reduced growth ability on ectoines as a carbon source (Figure 41). However, limited growth on ectoines as a carbon source was observed, similar to the growth behavior of *H. elongata* ΔdoeA (Figure 38).

Figure 41. Growth curves of *H. elongata* KB42 (ΔdoeB) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations.
5.4.3.13  *H. elongata KB43.1 (ΔdoeAΔectC)*

The deletion of *ectC* in the parent strain *H. elongata* KB43 (Δ*doeA*) resulted in a double mutant Δ*doeAΔectC* strain (*H. elongata* KB43.1) with no growth on ectoines. We did not even observe the limited level of growth seen in the parent strain KB43. In fact, the cell density tended to decline over time (Figure 38 Vs 42).

![Growth curves of *H. elongata* KB43.1 (Δ*doeAΔectC*) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations.](image)

Cultivation of *H. elongata* KB43.1 under conditions similar to those in figure 42 was performed on a larger scale (Figure 43). The screening of FMOC derivatized molecules allowed the detection of only γ-NADA (5) in cells from cultures from either ectoine or hydroxyectoine as a carbon source.
Figure 43. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB43.1 (ΔdoeAΔectC) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 5 = γ-NADA

5.4.3.14 *H. elongata* KB1.12 (ΔectAΔdoeA)

The deletion of doeA in the parent strain *H. elongata* KB1 (ΔectA) resulted in a double mutant strain KB1.12 (ΔectAΔdoeA) with no ability to utilize ectoines as a carbon source (Figure 44).
Cultivation of *H. elongata* KB1.12 (ΔectAΔdoeA) under conditions similar to those in figure 44 was performed on a larger scale (Figure 45). The screening of FMOC derivatized molecules resulted in the detection of glutamate (or 5) in cells from cultures with ectoine. No compounds were detected in the medium with hydroxyectoine. This case was similar to that observed in ΔdoeAΔeutC (Figure 39).

Figure 45. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB1.12 (ΔectAΔdoeA) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM).

5.4.3.15  *H. elongata* KB49.2 (ΔeutBΔdoeA)

The deletion of doeA in the parental strain *H. elongata* KB49 (ΔeutB) resulted in a complete elimination of the growth that has been previously observed in both single mutants when grown on ectoines as a carbon source (Figure 21 and 38 Vs 46). The growth curve on glucose-hydroxyectoine, however, clearly oscillated during the stationary phase. This growth behavior was somewhat similar to that observed in the case of strain *H. elongata* K2.3 (ΔeutBΔeutCectB::ΩΔdoeD) (Figure 33 Vs 46, triangles).
Results

Cultivation of *H. elongata* KB49.2 (ΔeutBΔdoeA) under conditions similar to those in figure 46 was performed on a larger scale (Figure 47). The screening of FMOC derivatized molecules allowed the detection of only glutamate (or 5) in cells from cultures with exogenous ectoine. No compounds were detected in the extract from culture with hydroxyectoine. The HPLC profiles were similar to those recorded for strain KB43.2 (Figure 40).

Figure 46. Growth curves of *H. elongata* KB49.2 (ΔeutBΔdoeA) when grown on MM63-6 (30 °C) in microplates obtaining carbon from different sources. Legend is depicted in the figure. G= glucose, G+E=glucose and ectoine, G+HE= glucose and hydroxyectoine. Concentrations at the beginning of the experiments: G=1 mM, E=HE=10 mM. Averages and standard deviations were calculated on data points free of medium background. Curves are the averages of two experiments in the cases of growth on glucose and averages of three experiments when ectoines were supplied in the media. Error bars represent the corresponding standard deviations.

Figure 47. HPLC profile of FMOC reactive metabolites extracted from *H. elongata* KB49.2 (ΔeutBΔdoeA) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). Glu = glutamate
5.4.3.16  \textit{H. elongata KB49.1.1 (ΔeutBΔectCΔdoeA)}

The deletion of \textit{doeA} in the parental strain \textit{H. elongata} KB49.1 (ΔeutBΔectC) resulted in the complete elimination of the growth that was previously observed in all three corresponding single mutants when grown on ectoines as a carbon source (Figures 21, 22 and 38 Vs 48). The growth behavior of strain KB49.1.1 was very similar to that observed in strain \textit{H. elongata} KB43.1 (ΔdoeAΔectC) (Figure 42 Vs 48).

Cultivation of \textit{H. elongata} KB49.1.1 under conditions similar to those in figure 48 was performed on a larger scale (Figure 49). The screening of FMOC derivatized molecules allowed the detection of only \textit{γ-NADA} (5) in cells from cultures with either ectoine or hydroxyectoine.
Results

Figure 49. HPLC profile of FMOC reactive metabolites extracted from H. elongata KB49.1.1 (ΔeutBΔectCΔdoeA) cells grown on media with two carbon sources. The growth curves in MM63-6 (30 °C) are plotted with cultivation time in the Z axis, with chromatograms rooting out in the X axis orientation according to the cultivation time at which the cells were harvested. The carbon sources were a) glucose (5 mM)–ectoine (15 mM) and b) glucose (5 mM)–hydroxyectoine (15 mM). 5= γ-NADA

5.4.3.17 Overview on the catabolism of ectoines in different H. elongata strains

In order to compare the effects of different mutations on the cell’s ability to utilize ectoines, the maximum growth rates of the different strains on both the glucose-ectoine and glucose-hydroxyectoine media (sections 5.4.3.1-16) were tabulated (Table 9). The first maximum growth rate (1st $\mu_{max}$) varied within a range of 0.27-0.47 h$^{-1}$ in ectoine-containing media and within 0.22-0.47 h$^{-1}$ in the hydroxyectoine-containing media. The highest maximum first growth rates in both media were observed in strains KB1 (ΔectA) and KB30 (ΔectR). The lowest maximum first growth rates were observed for strains KB49.2 (ΔeutBΔdoeA) and KB43.2 (ΔdoeAΔeutC). The second maximum growth rate (2nd $\mu_{max}$) varied within a range of <0.00-0.27 h$^{-1}$ for strains grown on ectoine as a carbon source and within a range of <0.00-0.17 h$^{-1}$ for strains grown on hydroxyectoine. The highest maximum second growth rates on ectoine as a carbon source were recorded for strains KB50 (ΔeutC) and KB13 (ΔectB); while the highest rates on hydroxyectoine were recorded for KB13 (ΔectB) and WUB01 (ΔectC). Thus, the single deletion of the biosynthesis genes ectB and ectC favored faster catabolism of ectoines relative to the wild type. Nevertheless, these strains took longer than the WT to reach the 2nd $\mu_{max}$ based on HE as C source (e.g. 0.17 h$^{-1}$ Vs 0.12 h$^{-1}$, for KB13 and the WT, respectively), reflecting the improvement in growth mentioned above. On the other hand, the deletion of ectA and ectR reduced strains’ ability to use ectoines as the single carbon source (e.g. 0.23 h$^{-1}$ Vs 0.16 h$^{-1}$, 2nd $\mu_{max}$ for WT and KB30, respectively). Despite this, the deletion of ectA and ectR favored rapid growth in the first phase.
Table 9. Maximum growth rates calculated for *H. elongata* strains growing in glucose-ectoines (MM63-6) media at 30 °C.

<table>
<thead>
<tr>
<th>Straina</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)b</th>
<th>$\Delta t_F$ (h)d</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)b</th>
<th>$\Delta t_{HE}$ (h)d</th>
</tr>
</thead>
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<tr>
<td></td>
<td>$1^{\text{st}}$ G+E</td>
<td>$2^{\text{nd}}$ E</td>
<td>$t_{\mu_{\text{max}}}$ G+E $t_{\mu_{\text{max}}}$ G+E</td>
<td>$t_{\mu_{\text{max}}}$ HE $t_{\mu_{\text{max}}}$ G+HE</td>
</tr>
<tr>
<td>$\Delta eutB\Delta doeA$</td>
<td>0.272 &lt; 0.000</td>
<td>ND$^d$</td>
<td>0.224 &lt; 0.000</td>
<td>ND$^d$</td>
</tr>
<tr>
<td>$\Delta eutA\Delta doeA$</td>
<td>0.281 &lt; 0.000</td>
<td>ND</td>
<td>0.239 &lt; 0.000</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta doeA\Delta eutC$</td>
<td>0.291 &lt; 0.000</td>
<td>ND</td>
<td>0.252 &lt; 0.000</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta eutB\Delta eutC\Delta doeA$</td>
<td>0.306 &lt; 0.000</td>
<td>ND</td>
<td>0.276 &lt; 0.000</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta doeA\Delta eutC$</td>
<td>0.269 &lt; 0.000</td>
<td>ND</td>
<td>0.219 &lt; 0.000</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta eutB\Delta eutC\text{::Ω}\Delta doeD$</td>
<td>0.329 0.221</td>
<td>6.500</td>
<td>0.277 0.000</td>
<td>ND</td>
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<tr>
<td>$\Delta doeB$</td>
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<td>ND</td>
<td>0.299 &lt; 0.000</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta doeA$</td>
<td>0.286 0.000</td>
<td>ND</td>
<td>0.306 0.000</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta doeD^c$</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>$\Delta eutR$</td>
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<td>7.500</td>
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<tr>
<td>$\Delta eutA$</td>
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<td>$\Delta eutC$</td>
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<td>6.000</td>
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<td>$\Delta eutB\Delta eutC$</td>
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<td>34.000</td>
</tr>
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<td>0.291 0.173</td>
<td>19.000</td>
</tr>
</tbody>
</table>

---

a Strains are ordered up to down by increasing magnitude of the maximum growth rate on hydroxyectoine as carbon source. When samples had similar values, reorganization was decided based on both, the first maximum growth rate and the adaptation time. The reference strain, the wild type, appears in green letters.

b Growth rates were calculated from average curves of three growth experiments (in microplates), the graphs are presented in sections 5.4.3.1-16. The $1^{\text{st}} \mu_{\text{max}}$ were calculated when cultures had available two carbon sources, glucose and one of the ectoines. The $2^{\text{nd}} \mu_{\text{max}}$ were calculated when cultures had only one of the ectoines for carbon source.

c From Schwibbert et al. (2011)

d The adaptation time ($\Delta t$) is a time proportional to the lag phase. It corresponds to the time required for the cells to change from the maximum growth rate in the presence of glucose to the maximum growth rate only on ectoines. The $\Delta t$ concept, as a representation of a factor reflecting the effect of a given mutation on the growth, was introduced because the diversity of behaviors resulting when the lag phases should occur difficult their measurement. The author recommends not to make conclusions based on adaptation time values alone, but to use it as an additional data to support conclusions based on different facts.

e ND=Not determined
6 Discussion

In order to study the conversion of hydroxyectoine to ectoine, I developed experiments specially designed to study this part of the metabolism in *Halomonas elongata*. The conversion situation outlined in section 5.1 suggested the participation of ectoine degradation genes because hydroxyectoine provided in the medium (5 mM) was not accumulated in the cytoplasm. Furthermore, the stationary phase occurred at a lower OD in the mutant **KB1** compared to the WT (~5 and ~7 respectively), indicating a lack of compatible solute instead of glucose depletion. The procedure used for section 5.1 was closely related to that used for section 5.4.2, where 13 strains were studied. Those findings demonstrate that the conversion of hydroxyectoine into ectoine was not completely abolished in any of the 13 strains when grown in glucose-hydroxyectoine media. Therefore, the discussion of the results obtained from experiments involving hydroxyectoine uptake will be often analyzed in terms of ectoine metabolism. Excluding the salinity, results in 5.1 and 5.4.2 were obtained in experimental conditions similar to those during the first growth phase of the diauxic growth experiments (results included in section 5.4.3). Therefore, they will mainly be discussed together in sections 6.1 and 6.2. The conversion of hydroxyectoine to ectoine is discussed in section 6.3 based on the conclusions made in previous sections and supported by the results related to the activity of recombinant proteins.

To facilitate the discussion, which includes many relationships, several of them among hypothetical unnamed intermediates, metabolites will be referred to by numbers that correspond to the molecular structures depicted in figure 4 (page 49). Numbers referring to metabolites will always be in bold.

6.1 Fluxes of ectoines’ carbons during growth phase in glucose-ectoines media

Following the discovery of the ectoines (Galinski et al., 1985, Inbar and Lapidot, 1988), many studies have focused on their biosynthesis in different organisms, but studies attempting to understand the catabolism of ectoines are still few. Most of what is currently known about the catabolism of ectoines has been garnered from observations made on *Sinorhizobium meliloti*. This soil bacterium, despite being able to
biosynthesize its own compatible solutes (glycine betaine, pipecolate, dimethyl sulfoniopropionate, sucrose, trehalose and other disaccharides) can also take up ectoine from the medium. However, once in the cytoplasm, ectoine continues to be broken down as a source of energy, rather than being maintained as osmoprotectant (Jebbar et al., 2005). The continued catabolization of ectoine even in the presence of glucose suggests that S. meliloti lacks a mechanism to regulate the cytoplasmic concentration of ectoine. This is not the case in C. salexigens, where the ectoine concentration is maintained, and its utilization as a carbon source is repressed by glucose, resulting in diauxic growth (Vargas et al., 2006). Most of the genes known to be involved in the catabolism of ectoine in S. meliloti (eutABCDE) have a homologous gene in C. salexigens that is also able to biosynthesize ectoines (as in H. elongata). Therefore, the differences in the fate of exogenously provided ectoine in the presence of glucose in these organisms indirectly suggest that ectoine biosynthesis genes might be involved in the regulation of ectoine concentration in the cytoplasm.

My main hypothesis was that the conversion of hydroxyectoine into ectoine may involve genes participating in both, the biosynthesis and the catabolism of ectoines (Figure 6). The proposed model for the utilization of ectoines included the cycling conversion of molecules \(6-7-4-5-6\) recently proposed by Schwibbert et al. (2011) as part of a mechanism to regulate the cytoplasmic ectoine concentration in H. elongata through simultaneous activity of the biosynthesis and the degradation pathways. The existence of this regulation cycle was indirectly supported by the fact that deletion of the gene doeA from a strain defective in the ectoine uptake system resulted in a \(\Delta\text{doeA}\) mutant (KB2.13) with increased volumetric ectoine productivity in 20 %.

The existence of this \((6-7-4-5-6)\) metabolite cycling for regulation of the ectoine level in the presence of glucose is further supported by the results of this study, in which the accumulation of \(7\) was noticed in \(\Delta\text{doeB}\) cells (H. elongata KB42) growing in minimal media that contained glucose (28 mM) supplemented with 5 mM hydroxyectoine (Figure 17). The level of \(7\) only increased significantly in the \(\Delta\text{doeB}\) mutant (KB42, figures 17), out of 13 strains examined [H. elongata strains DSM 2581\(^T\) (WT), KB43 (\(\Delta\text{doeA}\)), KB42 (\(\Delta\text{doeB}\)), KB49 (\(\Delta\text{eutB}\)), KB50 (\(\Delta\text{eutC}\)), WUB01 (\(\Delta\text{ectC}\)), KB43.1 (\(\Delta\text{doeA}\Delta\text{ectC}\)), KB43.2 (\(\Delta\text{doeA}\Delta\text{eutC}\)), KB49.1 (\(\Delta\text{eutB}\Delta\text{ectC}\)), KB49.1.1 (\(\Delta\text{eutB}\Delta\text{ectC}\Delta\text{doeA}\)), KB49.2 (\(\Delta\text{eutB}\Delta\text{doeA}\)), KB1 (\(\Delta\text{ectA}\)) and KB1.12 (\(\Delta\text{ectA}\Delta\text{doeA}\)). This suggests that in doeB\(^{+}\) cells, \(7\) is maintained at low levels due to the presence of DoeB in the cytoplasm.
Because *doeA* is transcribed together with *doeB*, we can conclude that DoeA is also present in the cytoplasm throughout the growth phase. This conclusion is further supported by the improvement in ectoine production reported by Schwibbert et al. (2011) for the Δ*doeA* strain (KB2.13) mentioned above. Important results from the analysis of these 13 strains (see section 5.4.2) also include the reduction of accumulated 5 in the cytoplasm of Δ*ectA* strains, KB1 and KB1.12 (Figure 19). This suggests that EctA is present in the cytoplasm of *ectA*+ strains and that it is involved in forming 5 in cells growing in HE supplemented media. Additionally, the accumulation of 5 in Δ*ectC* strains (WUB01, KB43.1, KB49.1 and KB49.1.1) is clear evidence of the presence of EctC in the cytoplasm of *ectC*+ strains. Also interesting is the fact that the Δ*doeA* strains (KB43, KB43.1, KB43.2, KB49.1.1, KB49.2, and KB1.12) could still accumulate low levels of 7, which implies the existence of an additional pathway allowing for the formation of 7. Screening for accumulation of 4 in these dividing cells detected only traces of it, even in Δ*ectA* strains (KB1 and KB1.12). This is not surprising considering that strain KB1 also showed poor accumulation of 4 while growing in glucose at 30 °C and 3 % NaCl (Schilz 2005). However, as none of the strains analyzed in this study accumulated significant amounts of 4 while growing in the presence of glucose, it is reasonable to conclude that significant regulation of the ectoine concentration is achieved via dilution in the increasing cytoplasm volume of growing cells. In this way, the flow of carbon from ectoine following path 6-7-4 is quickly addressed in direction 4-5(-6), thus limiting the flow of carbon in direction 4-3-2 (or 4-15). Nevertheless, complete elimination of carbon flow in direction 4-3-2-1 cannot be assured. Instead, since cultures in glucose-ectoine media reached a higher optical density at the end of the first growth phase (glucose depletion) than those grown on glucose–hydroxyectoine or only glucose (see section 5.4.3), a moderate flow of ectoine carbons along path 4-3-2-1 is likely. This observation is in agreement with the analysis of the fate of ectoine in the presence of glucose reported in *C. salexigens* by Vargas et al. (2006). In this study, a minority of radioactive carbons from labeled ectoine was found as part of respired carbon dioxide or macromolecules (<30 %).

One observation that was inconsistent with the hypothesis of an operating 6-7-4-5-6 metabolite cycling in the presence of glucose was the poor accumulation of 4 in the Δ*ectA* strain (data not shown). I suppose that the interruption of step 4-5 enhanced the
carbon flow in direction 4-3-2-1 by an unknown mechanism. This supposition is based on the fact that the ΔectA mutant (strain KB1) grown on glucose-ectoines media exhibited a first maximum growth rate on glucose-ectoine medium (0.46 h⁻¹) that was among the highest of the 16 strains studied. This growth rate was about 1.5 times that of the wild type (0.31 h⁻¹) and approximately twice that of the wild type grown on glucose-hydroxyectoine medium (0.24 h⁻¹). Additionally, there was no lag phase when the carbon source changed from glucose to ectoines (Figure 29). These facts are in agreement with the analysis of growth rates and ectoine uptake reported for an ectA defective C. salexigens strain by Vargas et al. (2006). The transposon mutant ectA::Tn1732 exhibited an increased ectoine uptake activity six-eight times higher than that registered for the corresponding WT when grown on glucose-ectoines media. The same study also proposed the simultaneous catabolization of both ectoines and glucose in this mutant. The proposed enhancement of metabolite flow in direction 4-3-2-1 in the H. elongata KB1 might also explain the results presented in section 5.1. Here it was shown that supplementation of the single mutant ΔectA with more hydroxyectoine (5 mM) than the ectoine accumulated in the wild type (equivalent to 3.5 mM) failed to support the maximum growth of ΔectA to the level observed in the WT at 11 % salinity (Figure 3). These observations suggest that the flow of carbon from ectoines into the central metabolism is proportional to the uptake activity. Thus, I propose that H. elongata KB1 (ΔectA), faces a metabolic situation similar to that observed in S. meliloti (but with no significant source of own compatible solutes) of continued degradation of ectoine with only partial use of it as osmoprotectant, due to a defect in the main mechanism to regulate the cytoplasmic ectoine concentration.
Based on the arguments presented above, the superior growth of *H. elongata* **KB1** (Δ*ectA*) relative to that of the **WT** in the glucose-hydroxyectoine medium at 6 % w/v NaCl (Table 9), but inferior growth in glucose-hydroxyectoine medium at 11 % w/v NaCl (Figure 3 and 15), could be explained by a deficient accumulation of ectoines due to their rapid flow into the central metabolism. Since poor accumulation of ectoines in the cytoplasm of the Δ*ectA* single mutant⁶ is required to support good growth at 6 % salinity, driving carbons from ectoines into the central metabolism must results in an enhancement of growth. On the other hand, at 11 % salinity, when the external osmotic pressure is about twice as great as that at 6 % salinity, driving the flow of carbon from ectoines into the central metabolism results in a loss of internal osmotic pressure and the consumption of a non-renewable source of internal osmotic pressure that prevents strain **KB1** from reaching the same maximum growth rate as the **WT**. Additionally, the relatively high accumulation of hydroxyectoine (regarding the ectoine pool) in the Δ*ectA* mutant (Figure 15) could be interpreted not as a poor conversion of hydroxyectoine to ectoine, but as an effect resulting from the enhanced uptake of hydroxyectoine and rapid flow of ectoines (especially the ectoine form from hydroxyectoine) into the central metabolism.

The same patterns of growth seen in the strain **KB1** (Δ*ectA*) were also observed in the strain **KB30** (Δ*ectR*) (Figure 27 Vs 29). This suggests that under the conditions of the experiments described in section 4.5.3 the Δ*ectR* mutant has a phenotype characterized by a deficiency of EctA. Therefore, I suggest that a function of EctR might be to activate the transcription of *ectA* in a regulated manner to allow a fine tuning of an ectoine level tending to an excess (in this case due to exceeding uptake), and being broken down by DoeA. Similar to strain **KB1** (Δ*ectA*), the mutant **KB30** (Δ*ectR*) did not accumulate 4 when glucose was still present while growing in glucose-ectoines media. This mutant also grew significantly better than the **WT** in this medium (about 1.5 times that of the **WT**: table 9). This can be explained by the repressed transcription of *ectA* in the presence of excess ectoines unless EctR activates its transcription.

⁶ *H. elongata* Δ*ectA* is able to growth at salinities up to 5 % without compatible solute supplementation, relaying possibly only in ADPC biosynthesis (Witt et al., 2011)
Vargas et al. (2006) suggested that the increased ectoine uptake activity displayed by the \textit{C. salexigens} transposon mutant \textit{ectA::Tn1732} might mean that endogenous ectoine in the WT could directly or indirectly repress its own uptake. The repression of ectoine uptake could in turn be explained by the release of the osmotic stress that might be involved in the triggering of the transcription and/or activity of the system to take up ectoines. In this study, similar phenotypes were registered for \textit{H. elongata} strains \textbf{KB1} and \textbf{KB30}, possibly due to the poor or deficient EctA biosynthesis in the presence of excess ectoines. Therefore, another possible explanation for the enhanced uptake activity and flow of ectoine carbon into the central metabolism is that EctA plays a direct or indirect role in the regulation of the ectoine uptake system (i.e. inhibition) and in the use of ectoine as a carbon source (i.e. inhibition). Another possibility is that those roles are played by EctR, but that seems less probable because deletion of \textit{ectA} is unlikely to affect the transcription of \textit{ectR}, especially considering that the strain \textbf{KB1} can make ADPC expressing EctC (Witt et al., 2011). Also, \textit{H. elongata} \textbf{KB1.12} (\textit{ΔectA\&ΔdoeA}) grows more poorly than the strain \textbf{KB1} (\textit{ΔectA}) in a glucose medium with hydroxyectoine as a compatible solute supplement at 11 % salinity (Figure 15a), in contrast to the hypothesis above. The \textit{ΔdoeA} mutation is expected to significantly reduce the cleavage of ectoines, leading to an increase in the accumulation of compatible solutes and to increase the intracellular osmotic pressure that should be reflected in a better growth of strain \textbf{KB1.12} relative to strain \textbf{KB1}. With this in mind, the observation that a mutation in \textit{ectA} caused an enhancement in the uptake was surprising. This observation was made in \textit{C. salexigens} and was also supported indirectly by the results herein reported for \textit{H. elongata} KB1 (\textit{ΔectA}). However, it could be that the \textit{ΔdoeA} mutation caused an enhancement in the activity of the unknown efflux system, either directly or indirectly. This in addition to fit with the results would provide another possible explanation for the improvement in the productivity observed by Schwibbert et al. (2011).

The proposed function of EctR as a transcription activator of \textit{ectA} in the presence of excess ectoines might help to understand the results reported by Schilz (2005). Schilz compared strain \textbf{KB30} (\textit{ΔectR}) to \textbf{KB1} (\textit{ΔectA}), and during the growth phase\textsuperscript{7} provided a relatively weak pulse of ectoine (1 mM) accompanied by a salt shock (1-10 %). In

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\textsuperscript{7} In this study we have proposed that regulation of the ectoine abundance during growth on glucose is also achieved by dilution in the growing cytoplasm.
agreement with this study, shortly after providing a pulse of 1 mM ectoine, growth of KB1 and KB30 (ΔectA and ΔectR, respectively) improved, but only for a short period (<1h). Combining ΔectR and ΔectA in one mutant did not help to find the phenotype. Although the growth of strain KB30 (ΔectR) was different than that of the WT at different salinities, the FMOC-HPLC analysis of amino-reactive metabolites extracted from cells grown on glucose at 3 % salinity showed the same composition, with no new metabolites. Glutamate was the major metabolite and only traces of 2,4-diaminobutyric acid (4) were found. Based on these results it was difficult to draw conclusions regarding the role of EctR. The function proposed for EctR in the present study also agrees with its putative function as a positive transcription regulator (transcription activator), as was inferred from a computer analysis of the amino acid sequence (Stumpfe 2003).

6.2 The lag phase of the diauxic growth and the catabolism of ectoines

The deficiency of growth due to glucose depletion in glucose-ectoine media prevents dilution of uptaken ectoines in an increasing cytoplasm volume. This implies that the regulation of the cytoplasmic ectoine concentration relies mainly on controlling uptake activity and the 6-7-4-5-6 metabolite cycling, which results in the accumulation of 4 during the lag phase of the diauxic growth experiments (section 5.4.3), as was observed in six H. elongata strains: WUB01 (ΔectC), KB50 (ΔeutC), KB30 (ΔectR), KB1 (ΔectA), KB49.1 (ΔeutBΔectC), K2.3 (ΔeutBΔeutCectB::ΩdoeD), figure 23, 25, 28, 30, 32 and 34, respectively. The accumulation of 4 in these strains indicates that the mechanisms to modify 4 (possibly dependent on ectA, ectB and maybe others) were down regulated as glucose was depleted and ectoine was accumulated (or that DoeB activity increased, or both). Nevertheless, accumulation of 4 could not be observed in H. elongata strains KB43.2 (ΔdoeAΔeutC), KB43.1 (ΔdoeAΔectC), KB1.12 (ΔectAΔdoeA), KB49.2 (ΔeutBΔdoeA), and KB49.1.1 (ΔeutBΔectCΔdoeA) as shown in figures 39, 43, 45, 47 and 49. This demonstrates that the main source for accumulation of 4 (via DoeB) in the six strains mentioned above requires the participation of DoeA.

In this section an effort to explain the flow of carbons is made base on 16 strains. The generalized conclusions made on this analysis are, however, only rarely applicable to strains ΔectA and ΔectR, which often behave exceptionally.

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The medium used to study the diauxic growth provided cells with an abundance of ectoine. Therefore, accumulation of 4 in the lag phase indicates a limiting flow of ectoine carbons into the citric acid cycle, which is possible in the absence of DoeC (Schwibbert et al. 2011). Consequently, ectoine tends to exceed an “ideal optimal concentration” to counterbalance the external osmotic pressure. Hence, the accumulation of 4 by down-regulation of ectAB is interpreted as a strategy of H. elongata cells to “buffer” the cytoplasmic concentration of ectoine. However, since 4 is not accumulated while cells are dividing in the presence of glucose, down-regulation of ectAB should not be understood as complete repression. Instead, a regulated transcription of ectAB(C) can explain the mechanism maintaining ectoine concentration at an appropriate level.

The lag phase was observed in the different strains as the maximum optical cell density reached in the presence of glucose. It did not occur at the same optical density in all of the 16 strains analyzed. This seemed to be related to three factors: the repression of ectoine synthesis (flow of glucose carbons in direction 1-2-3-4-5-6), the cell’s ability to use ectoines as a carbon source in the presence of glucose (i.e. the flowing of the ectoines’ carbons in direction 6-7-4-3-2-1), and the ability to regulate the cytoplasmic ectoine level (cycling of carbons among molecules 6-7-4-5-6). The repression of the synthesis of ectoines using glucose carbons in cultures supplemented with ectoines would surely increase the amount of glucose available for growth, resulting in an increased first maximum optical cell density (>0.21) relative to non-supplemented cultures (first maximum OD 0.12-0.17), as observed in the growth behaviors of all 16 strains studied (section 5.5.3). In hydroxyectoine supplemented cultures the first maximum growth occurred within a narrow OD range (0.21-0.25). Conversely, cultures grown in ectoine supplemented medium varied in their maximum OD (0.21-0.35).

Therefore, the ability of strains to cycle carbon among molecules 6-7-4-5-6 is proposed as another factor influencing the cell density at which the lag phase occurs. The maximum OD achieved during the end of the first growth phase in cultures grown in glucose-ectoine media occurred in strains with full cycling ability (i.e. WT, figure 20; and KB49, figure 21, OD~0.35) and the minimum OD in strains with limited cycling ability (i.e. KB1 = KB30 < WUB01; figures 30 Vs 28 Vs 23; OD~ 0.23, 0.25 and 0.21, respectively). The strains with limited cycling activity displayed an enhanced but deleterious and unregulated buffering of the cytoplasmic ectoine concentration. The fact that the maximum OD defining the end of the first growth phase was so variable, mainly
among ectoine containing cultures, could be explained by the fact that the uptake of ectoine is more efficient than the uptake of hydroxyectoine (Kuhlmann et al., 2002). Therefore, the ability to cycle molecules 6-7-4-5-6 seems to be more important in a cytoplasm tending towards ectoine saturation and would eventually facilitate flow of ectoine carbons in direction 6-7-4-3-2-1. This would result in a further increment of the OD achieved in the presence of glucose. The previous hypothesis is supported by the fact that cultures of strains that were completely unable to grow on ectoine as carbon source (i.e. KB43.1 (ΔdoeAΔectC), KB1.12 (ΔectAΔdoeA), KB49.2 (ΔeutBΔdoeA), and KB49.1.1 (ΔeutBΔectCΔdoeA) (figures 43, 45, 47 and 49, respectively)), achieved equal maximum growth on glucose-ectoine medium to those grown in glucose-hydroxyectoine medium (~0.21). The absence of growth on ectoines as a carbon source in these strains shows that they have blocked all pathways to send carbons from ectoines into the central metabolism. Therefore, if no additional degradation pathways are used in the presence of glucose, the maximum growth of these strains represents the upper limit they can reach based on glucose. This can be explained by the maximized utilization of glucose caused by an ectoines-induced repression of the glucose-derived carbon flow in direction 1-2-3-4-5-6. Furthermore, it implies that in cells of cultures supplemented with hydroxyectoine, the flow of hydroxyectoine carbon into the central metabolism does not occur. Therefore, the growth observed in ectoine-supplemented cultures beyond the maximum OD achieved by cultures growing in hydroxyectoine-supplemented medium is explained as a result of a flow of ectoine carbons into the central metabolism.

The time period required to adapt the cell’s metabolism from using glucose to ectoines as a carbon source (lag phase extension) might be related, among other factors, to the ability of a particular strain to buffer ectoine concentration when it is at high levels (see above). Since the uptake system is more efficient with ectoine than with hydroxyectoine, the lag phase to change from glucose to ectoine is shorter than that required to switch from glucose to hydroxyectoine. Consequently, expression of the critical genes required to grow on ectoine as a sole carbon source (i.e. genes doeA, doeB and doeC (as could be deduced from growth experiments: figures 36 and 47, and from Schwibbert et al. 2011), should be relatively easy to activate in excess ectoine concentration. Therefore, the effects of non-critical mutations on the growth phenotype are too difficult to differentiate during the short lag phase for switching from glucose to ectoine as a carbon source. On the other hand, the effects of non critical genes on the
catabolism of hydroxyectoine can be observed as a prolonged lag phase when changing from glucose to hydroxyectoine as a carbon source. Hence, although \( \text{eutC} \) is not essential for the catabolism of HE, its importance is evident in the growth curve of strain \( \text{KB50 (}\Delta\text{eutC}) \) by the clearly extended lag phase. This was followed by reduced growth on HE as a carbon source (Figure 24, triangles). Genes \( \text{eutB} \) and \( \text{ectC} \) seem to be less important, but if both are deleted from the same strain (\( \text{KB49.1:} \Delta\text{eutB}\Delta\text{ectC} \)), this also leads to a significant defect in the metabolism of HE (Figure 31, triangles). This could be because there is more than one active branched path for hydroxyectoine degradation, and both pathways are temporarily affected by the inactivation of these two genes. However, more genes must exist to complement the observed deficiency because growth recovered later in the cultivation period.

6.3 Hydroxyectoine conversion to ectoine

The study of the \textit{in vivo activity} of DoeA revealed its ability to cleave hydroxyectoine, apparently following the same mechanism that is used to cleave ectoine (Figures 7 and 8). Because DoeA and DoeB are in the cytoplasm of cells supplemented with HE, the proposed pathway to form 11: 8\(-10\)-11 (Figure 50), would not be surprising. This conversion is also supported by the fact that metabolite 11 is accumulated in strain \( \text{K2.3 (}\Delta\text{eutB}\Delta\text{eutCectB::}\Omega\Delta\text{doeD})} \) (Figures 34 and 36), which presented severe deficiencies in the utilization of HE (8) as a sole carbon source (Figure 33). Formation of 11 from 8 requires a deacetylation step. Two enzymes are known to have acylase activity in the metabolism of ectoines, namely EctA and DoeB. However, only DoeB has been shown to have degradation activity. Therefore, although a low degree of reversibility by EctA cannot be ruled out, the formation of 11 is more likely to follow step 10\(-11\) mediated by DoeB. The reduced metabolization of 11 in strain \( \text{K2.3} \) implies that, under these particular experimental conditions (8 as a carbon source in the absence of glucose), the main means to avoid accumulation of 11 do not operate properly (see figure 50).

Interestingly, the difficulty observed in strain \( \text{K2.3} \) to utilize 8 did not influence the growth on 6 as a carbon source. Schwibbert et al. (2011) showed that deletion of both \( \text{ectB} \) and \( \text{doeD} \) did not stop biosynthesis nor degradation of ectoine, and therefore deduced that there must be other enzymes catalyzing step 3\(-4\). However, since growth on 8 was severely affected in strain \( \text{K2.3} \), it is unlikely that these unknown enzymes play a role in proposed step 11\(-12\) (in the absence of glucose). Conversely, if the
metabolism of 11 continues to form 12, accumulation of 11 shows that either DoeD or EctB (or both) is very important to catalyze this conversion. Since only traces of 11 could be detected in cells of strain K2.3 in the presence of glucose, it is possible that the conversion 11-15 (and 12-14) is catalyzed by dehydratases other than EutB that are expressed mainly in the presence of glucose. During the first growth phase, the regulated transcription of ectA might lead to an enhancement of conversion 11-9, thereby avoiding the accumulation of 11 (assuming that EctA like DoeA and EctC can act on the 3-hydroxylated derivatives of their corresponding known substrates too). The accumulation of 11 in nondividing (or poorly dividing) K2.3 cells might also have been promoted by an enhancement in the uptake of 8, which is likely to be partially repressed in the presence of glucose. This is a complicated situation for strain K2.3 because of a disabled EutC-mediated pathway for utilization of 8 (see below). These facts strongly suggest that EutB is the main dehydratase for the utilization of 8, catalyzing at least one of the proposed hypothetical conversions of intermediates 17-18, 9-16 and 11-15.

Figure 50. Hydroxyectoine metabolism from figure 4 depicting the mutations present in strain K2.3 (ΔeutBΔeutCectB::ΩΔdoeD). Shown is the accumulation of the novel metabolite 2,4-diamino-3-hydroxybutyric acid (11) in a K2.3 background while mutant cells try to use HE (8) as carbon source. Red line goes over arrows corresponding to hypothetical enzymatic steps possibly defective due to mutations.

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_H. elongata_ strain **KB50** (ΔeutC) also underwent a remarkable reduction in its ability to grow on 8 (Figures 24 and 25b). Therefore, it is reasonable to think that it allows the main pathway for utilization of 8 as a sole carbon source. Moreover, accumulation of six-carbon intermediates in this strain was not observed. This is in agreement with the proposed function for EutC of catalyzing conversions 8-17. This hypothetical conversion might also proceed to form an alpha N-acetylated version of 17. The extended lag phase displayed by strain **KB50** to achieve growth on hydroxyectoine is therefore likely to be related to the optimization of secondary pathways (e.g. 8-9 or 8-10, or gene complementation for 8-17), for example by increasing the expression of DoeA. The ΔeutC strain showed, however, only poor accumulation of 11 during the lag phase, during which time it accumulated mainly 4. Contrary to strain **K2.3**, **KB50** (ΔeutC) had active _eutB_, _doeD_ and _ectB_ and so it is not surprising that most of the 11 formed would have been rapidly converted into 4 following paths 11-15-4 or 11-12-14-3-4. The broad peak displayed in the chromatogram of **KB50** (ΔeutC) (Figure 25b, 15-20 min) might consequently correspond to intermediate 12. This hypothesis is supported by the fact that this broad peak is absent in strain **K2.3** (defective in conversion 11-12), which accumulated the precursor of 12, 11. If conversion 11-9 or 4-5 had been occurring, the amounts of 9 and 5 formed would not have been sufficient to accumulate to such a remarkable level (Figure 25b). This strongly suggest a low abundance of EctA in the cytoplasm of **KB50** (ΔeutC) cells during the lag phase in order to adapt the metabolism for utilization of 8 as the sole carbon source. This hypothesis is strongly supported by the fact that the intermediate accumulation in strain **KB50** (ΔeutC) cells followed the same patterns as in strain **KB1** (ΔectA) when grown on hydroxyectoine (Figure 25b Vs 30b). However, instead of decreasing the conversion of hydroxyectoine to ectoine in the presence of glucose, the ΔeutC mutation did favor this conversion (Figure 14). This shows that the ΔeutC mutation alone, despite of its importance in the catabolism of 8, has minor effects on the conversion 8-6 in the presence of glucose.

The analysis of hydroxyectoine cleavage during the _in vivo_ activity test in the system carrying genes _eutBC_ (Figures 6-8) did not provide any conclusive evidence of cleavage activity on 6 or 8 by the corresponding enzymes. The presence of EutB and EutC in the cytoplasm of _E. coli_ BL21 pET22b_eutBC cells was only shown ambiguously and it is possible that EutC was not present at all. However, without an active ectoines-cleaving enzyme (e.g. EutC and DoeA) no activity of EutB is to be expected.
Therefore, a strong conclusion would be that the EutB enzyme is unable to cleave the ectoines’ ring. Nonetheless, strains carrying the eutBC sequence grew better with EutB than without it. This indicates that an over-expressed protein somehow enhances the growth ability of E. coli. This might be due to the activity of EutB over a native metabolite biosynthesize by E. coli. This fact, and the fact that this system yielded more biomass than the strains carrying an empty plasmid, will not be discussed further because without definite results, further interpretation would be purely speculative.

The fact that K2.3 cells could not grow on 8, accumulated 11, 4 and relatively small amounts of 6, makes it difficult to decipher the origin of the accumulated 4. However, the abundance of 6 was notably reduced even in the presence of glucose (Figure 35 and data not shown). Strain K2.3 had no problem growing on 6 as a carbon source. This proves that its 6-7-4-3-2-1 conversion ability is effective. The unusual oscillating average growth curve of K2.3 on 8 as the sole carbon source might be explained as the result of a poor 8-(4, 7 or 6) conversion ability that is not totally eliminated but that occurs moderately, eventually providing enough accumulation of the right intermediates (e.g. 4, 7 or 6) to generate pulses of growth. It is also remarkable that this strain, by accumulating both 4 and 11, buffered ectoines to higher levels, which provided additional difficulties for the utilization of 8 as a carbon source (see section 6.2).

H. elongata KB49.1 (ΔeutBΔectC) is another strain that was able to grow on hydroxyectoine, but experienced difficulties (Figure 31). It was discussed above the significance of EutB for the metabolism of 8 based mainly on the results from strain K2.3. However, the deletion of eutB alone caused only a relatively short extension of the lag phase to change the carbon source from glucose to 8 (Figure 21) and caused minor difficulties in converting 8--6 (Figure 14). Hence, in the presence of glucose, the deletion of eutB alone has minor effects on the metabolism of 8. The proposed role of EutB in catalyzing the steps 17-18, 9-16 and/or 11-15 while growing on 8 as a carbon source is probably compensated for in the single mutant ΔeutB (KB49) by dehydroxylating 12, 13 or 17 in conversions 12-14, 13-1 or 17-18. This reaction could be catalyzed by an unknown dehydratase (e.g. threonine dehydratase) that could have a broader specificity. On the other hand, the ΔectC mutation is crucial for an effective conversion 8--6 (Figure 15), but not for the utilization of 8 as sole carbon source (Figure 22). The introduction of the ΔectC mutation in H. elongata KB49 (ΔeutB) resulted in a strain (KB49.1) with the most limited ability to form 6 by conversion 8--6 (Figure 15b).
Therefore, it is reasonable to conclude that these two enzymes, EutB and EctC, contribute to the 8--6 conversion through at least one different pathway. Complementation by a dehydratase other than EutB did not restore the conversion ability to the level observed in the strains with the single mutations ΔectC or ΔeutB, suggesting that the unknown dehydratase mainly provides EctC with 5, while EutB must provide substrate to EctC and to an ectoine synthase other than EctC (e.g. DoeA or EutC). The extended lag phase observed with strain KB49.1 (ΔeutBΔectC) to change from glucose to 8 as a carbon source, should consequently be associated with an enhancement of its ability to buffer ectoine relative to the single mutant ΔeutB. This was accompanied by the typical accumulation of 5 caused by the ΔectC mutation, which could have hampered the expression of the unknown dehydratase to complement EutB activity.

Since DoeB has been shown in vitro to be a relatively strong enzyme acting on conversion 7-4 (Guo, J., Schmidt, S., Schwibbert, K. and Kunte, H.J., unpublished results) it is expected that its activity would pull the equilibriums of the hydroxylated intermediates down to 11 and force the main flow of hydroxyectoine carbons to follow the pathway 8-10-11-15-4-5-6 (or 8-10-11-12-14-3-4-5-6) for the conversion to ectoine. These proposed conversion pathways (8 via 10 to 11) are supported by the results from the feeding experiments presented in section 5.1, where strain KB1 (ΔectA) supplemented with hydroxyectoine used the latter as a carbon source instead of using it exclusively as osmoprotectant. Obviously, once metabolites 4 or 11 are formed, in the absence of EctA, there is no way to reuse their carbons for biosynthesis of 6 and 8. Hydroxyectoine conversion to ectoine should follow deviations from the main pathway with relatively few complications because it was demonstrated that strains that were defective in steps 8-10 (ΔdoeA), 10-11 (ΔdoeB), or 4-5 (ΔectA) still did well in the conversion. However, strains experienced more serious difficulties if the step 5-6 (ΔectC) was defective. The system is even more complex because the preferred path (or the enzymes catalyzing them) in a given stage of growth seems to depend, among other factors, on the carbon source available.

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10 Additional details about the ΔectA mutants were discussed in section 6.1.
6.4 Significance of the study

The analysis of the conversion of hydroxyectoine to ectoine led to the development of a model that is based on the oxaloacetate in the citric acid cycle. It considers eight metabolites (1-8) currently known to take part in the biosynthesis and degradation pathways of ectoines (Figure 2). The model was extended to include 11 hypothetical intermediates (9-19) linked through steps supported by the known and predicted activity of enzymes with the potential to catalyze them. This model proved to be useful in *H. elongata* in guiding the design of key experiments to significantly advance in the understanding of the metabolism of ectoines. Its use might be extended to other organisms; even those incapable of *de novo* biosynthesis of ectoines. A good example would be an *E. coli* strain bearing an ectoine operon that could be analyzed for recombinant protein-mediated ectoine biosynthesis, or genetically engineered for the degradation of ectoines. The application of the ectoines’ model is of particular importance in this bacterium because the present study demonstrated that the *E. coli* genome codes for enzymes that directly influence conversions among molecules DA (4), \(\gamma\)-NADA (5) and \(\alpha\)-NADA (7); and most probably among molecules \(\gamma\)-NA-HO-DA (9), \(\alpha\)-NA-HO-DA (10) and HO-DA (11). This means that an EctA, EctB, EctC, DoeA, DoeB or a DoeD expressed in *E. coli* faces substrate competition with at least one native enzyme.

The strategy applied in previous studies to assess the phenotype of *H. elongata* strains and related bacteria was optimized including as major variable the bacterial cultivation in microplates. The optimized strategy provided results that demonstrate its reliability, reproducibility, and superior resolution. At the same time, it is an approach that simplifies the comparison of several mutants simultaneously. The main illustration of the success of this technique was the identification of the strain *H. elongata* K2.3 as a key strain to understand the catabolism of hydroxyectoine. Additional important contributions of this study are:
• The collection of phenotypic evidence that relates the genes *eutB* and *eutC* with the metabolism of ectoines, especially to the utilization of hydroxyectoine as the sole carbon source.

• The collection of phenotypic evidence that relates the previously unsuccessfully studied gene *ectR* with the expression of EctA. The proposed function for EctR is as a transcription activator of *ectA* in a cytoplasm tending to excessive concentration of ectoines.

Accumulation of high levels of the novel intermediate (2,4-diamino-3-hydroxybutyric acid) was observed in *H. elongata* K2.3 when hydroxyectoine was the only carbon source available. This metabolite was also observed in other strains when the expression of EctA or EctC was defective, but only accumulated to a limited extent. This novel metabolite was placed in the model in a putative degradation pathway for hydroxyectoine. The hypothetical correlations proposed in the model for the novel metabolite fit all four mutations present in strain K2.3 and justify its accumulation. The model suggests that the novel metabolite could continue to be degraded as a carbon source to reach the citric acid cycle via three steps including, as a final step, the conversion of hydroxyaspartic acid to oxaloacetate. By avoiding the expression of DoeC and DoeD, it might also be used to form ectoine favoring a very likely conversion pathway.

The introduction section is a critical summary of osmoadaptation, including the results of over 20 years of research. The degradation of hydroxyectoine has received less attention to-date, and appears to have been more difficult to study, leading to fewer published data on that topic. This study (<4 years) cannot resolve all of the complex regulation mechanisms affecting the accumulation of hydroxyectoine. However, it does provide a starting point for future research on this largely unknown part of the metabolism of hydroxyectoine.
7 Conclusion

The genes *eutB*, *eutC* and *doeA* of *H. elongata* were identified as potentially participating in the conversion of hydroxyectoine to ectoine. By studying the *in vitro* activity of the recombinant proteins as part of the cytoplasm of *E. coli*, only DoeA could be proved to modify the structure of hydroxyectoine. Nevertheless, the analysis of the phenotypes of deletion mutants defective in genes *eutB* and *eutC*, supported their participation in the catabolism of hydroxyectoine.

A model showing the pathways for the biosynthesis and degradation of ectoines in combination with hypothetical pathways for hydroxyectoine conversion to ectoine was constructed. By linking the hypothetical degradation intermediates and the biosynthesis of hydroxyectoine, the model led to the identification of several enzymatic steps of relevance in the conversion pathway. Based on this input, seven mutant strains were created (Δ*eutC*, Δ*doeAΔectC*, Δ*doeAΔeutC*, Δ*eutBΔectC*, Δ*eutBΔectCΔdoeA*, Δ*eutBΔdoeA* and Δ*ectAΔdoeA*), and the study was extended to include 16 *H. elongata* strains. This approach allowed us to demonstrate the participation of genetic elements, along both biosynthesis and degradation pathways, in the conversion of hydroxyectoine to ectoine.

The establishment of a new strategy to phenotype *H. elongata* strains by cultivation in media containing two carbon sources in microplates facilitated the reliable comparison of several strains. The phenotypic analysis revealed a diauxic growth as normal behavior when switching from glucose to an alternative ectoines-carbon source. Some degree of simultaneous metabolism was concluded in the case of cultures growing in glucose-ectoine, but the same could not be concluded for cultures growing in glucose-hydroxyectoine media.

The accumulation of intermediates during glucose-ectoines diauxic growth experiments was shown to be related to the growth phase and the carbon source available. Accumulation of 2,4-diaminobutyric acid was found to be typical of the lag phase to switch from glucose to ectoines as a carbon source. This may be part of a mechanism
used by *H. elongata* cells to regulate the cytoplasmic level of ectoines when it is excessive, for example while cell division is limited.

Based on the phenotypic results, in combination with the accumulation of intermediates, a function for EctR is proposed in this study. The proposed function for EctR is as a transcription activator of *ectA* in a cytoplasm tending to excessive concentration of ectoines.

Analysis of the accumulation of intermediates as a result of hydroxyectoine metabolism in several mutant strains led to the detection of a novel metabolite identified as 2,4-diamino-3-hydroxybutyric acid. This metabolite accumulated at low levels and only in the absence of glucose in strains with a defective ectoine regulation cycle. It was, however, found as a major FMOC amino-reactive metabolite in strain **K2.3** (*ΔeutBΔeutCectB::ΩΔdoeD*). This strain could utilize ectoine as a carbon source, but utilization of hydroxyectoine was seriously hindered.

This study demonstrated that the hydroxyectoine conversion to ectoine involves complex patterns of gene regulation due to catabolite repression and gene complementation. The hydroxyectoine structure must be also a substrate of a ring cleaving enzyme different than DoeA. Dehydration of hydroxyectoine-derived intermediates catalyzed by EutB was not demonstrated but this role is strongly suggested by the phenotypic data. However, there must be at least one more dehydratase participating in the conversion of hydroxyectoine to ectoine. In the absence of glucose, EutB seems to be the most important dehydratase.

The mutants constructed during this study were investigated in the context of the objectives of this research. However there are many other interesting aspects of them still unknown, including for example their potential contribution to improving the production of ectoine or hydroxyectoine. Therefore, the extension of the impact of this research cannot yet fully be defined. However, this works opens a window from which scientists in the field can gain new insights into the metabolism of ectoines, and I hope to motivate others to focus on the topic.
8 References


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9.3 Index of Abbreviations

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>≈</td>
<td>Approximated or approximately</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>::</td>
<td>DNA sequence insertion</td>
</tr>
<tr>
<td>δ</td>
<td>NMR chemical shift (ppm)</td>
</tr>
<tr>
<td>% v/v</td>
<td>Volume volume percentage</td>
</tr>
<tr>
<td>% w/v</td>
<td>Weight volume percentage</td>
</tr>
<tr>
<td>aa.</td>
<td>Amino acid</td>
</tr>
<tr>
<td>amu</td>
<td>Atomic mass units</td>
</tr>
<tr>
<td>AB</td>
<td>Antibiotic Broth Nr. 3 (Medium)</td>
</tr>
<tr>
<td>ADAM</td>
<td>1-Aminoadamantan</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>AS</td>
<td>Aspartate semialdehyde</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>b</td>
<td>Bases</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BRENDA</td>
<td>BRaunschweig ENzyme DAtabase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumine (Rinderserumalbumin)</td>
</tr>
<tr>
<td>C.</td>
<td><em>Chromohalobacter</em></td>
</tr>
<tr>
<td>Δ</td>
<td>Deletion</td>
</tr>
<tr>
<td>DA</td>
<td>2,4-diaminobutyric acid</td>
</tr>
<tr>
<td>HO-DA</td>
<td>2,4-diamino-3-hydroxybutyric acid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>de</td>
<td>Demineralized</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>doe (ABXCD)</td>
<td>Genes involved in the degradation of ectoines (see figure 1)</td>
</tr>
<tr>
<td>Doe (ABXCD)</td>
<td>Enzymes involved in the degradation of ectoines (see figure 2)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Desoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DSM</td>
<td>Deutsche Stammsammlung für Mikroorganismen</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Ectoine</td>
</tr>
<tr>
<td>E.</td>
<td><em>Escherichia</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>For example</td>
</tr>
<tr>
<td>et al.</td>
<td>And co-authors</td>
</tr>
<tr>
<td>ect (ABCRD)</td>
<td>Genes involved in the biosynthesis of ectoines (see figure 1)</td>
</tr>
<tr>
<td>Ect (ABCRD)</td>
<td>Enzymes involved in the biosynthesis of ectoines (see figure 2)</td>
</tr>
<tr>
<td>eut (BC)</td>
<td>Genes putatively involved in the utilization of ectoines (see figure 1)</td>
</tr>
<tr>
<td>Eut (BC)</td>
<td>Enzymes putatively involved in the utilization of ectoines (see figure 2)</td>
</tr>
<tr>
<td>FMOC</td>
<td>9-Fluorenylmethoxycarbonyl chloride</td>
</tr>
<tr>
<td>G</td>
<td>Glucose</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
</tbody>
</table>

Units of the international system and simple derivative ones are not listed.
Appendix

i.e. That is
H. *Halomonas*
HE Hydroxyectoine
HO- Hydroxy-
HPLC High performance or high pressure liquid chromatography
IPTG Isopropyl-1-thio-β-D-galaktopyranosid
KCl Potassium chloride
Kan Kanamycin
LB Luria-Bertani (-Medium)
LBG Luria-Bertani-Medium with Glucose
MeCN Acetonitrile
MM63 Minimal medium 63
NADA N-acetyl-2,4-diaminobutyric acid
NA-HO-DA N-acetyl-2,4-diamino-3-hydroxybutyric acid
NaCl Sodium chloride
MHz Megahertz
Msc Mechanosensitive channel
MW Molecular weight
ND Not detected, not determined
NMR Nuclear magnetic resonance
OD Optical density, subscripts refer to the wavelength (600 nm unless otherwise specified)
OH- Hydroxy-
OPA O-phtalaldehyde
p Plasmid
P(1,2,3) German: Puffer = buffer(1,2,3)
PAGE Polyacrylamide gel electrophoresis
PCR Polymerase chain reaction
pH -log [H₃O⁺]
ppm Parts per million
RBS Ribosome binding site
RNaseA Ribonuclease A
RP Reversed phase
rpm Revolutions per minutes
RSB Reducing sample buffer
RT Room temperature
S. *Sinorhizobium*
SDS Sodium dodecylsulfate
SOE Splicing by Overlap Extension
sp. Species
Taq *Thermus aquaticus*
tea (ABCD) Transporter for ectoine accumulation (see figure 1)
Tea (ABCD) Enzymes involved in the transport for ectoine accumulation (see figure 1)
TES N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TEMED N,N,N',N'-Tetramethylethylene diamine
THF Tetrahydrofuran
tᵣ Retention time
Tris Trihydroxymethylaminomethane
UV Ultraviolet
U Unit
UV Ultraviolet
V Volt
WT Wild type
4.6. Additional figures

Figure 51. Sequence alignment to verify the inserted DNA sequence in the expression vector pET-22b _eutBC_. Region 5095-7294 b (5’→3’) in black letters. DNA sequences experimentally determined are presented in different colors according to the primers used for their amplification: T7-promoter primer, brown letters; Seq3660-F2 primer, blue letters; Seq3659-F2 primer: orange letters and T7-terminator primer: purple letters. Restriction sites are underlined and followed by a superscript letter that indicates the corresponding restriction enzyme: N= _Nde_ I, H= _Hind_ III.

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Figure 52. Sequence alignment to verify the inserted DNA sequence in expression vectors with doeA gene. a) Region 5101-6496 of pET-22b does in black letters (5’→3’) and b) Region 5301-6599 b of pET-15b: doeA (his)_N is in black letters (5’→3’). DNA sequences experimentally determined are presented in different colors according to the primers used for their amplification: T7-promoter primer, brown letters and T7-terminator primer in purple letters. Restriction sites are underlined and followed by a superscript letter that indicates the corresponding restriction enzyme: N= NdeI, H= HindIII.
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Figure 54. SDS-PAGE profiles of proteins mixtures extracted from *E. coli* BL21 cells harvested during an over-expression experiment to analyze the toxicity of target genes and the expression of target proteins. Lanes are labeled from top to down with a letter indicating the type of plasmids carried by corresponding cells sample (A = pET-22b_doeA or BC pET-22b_eutBC), a letter indicating the compatible solute supplementing the cells (E = ectoine; HE = hydroxyectoine) and the time after induction at which the cells were harvested. Samples correspond to growth curves in figure 49. Areas in the squares show protein bands due to basal expression. Lane on the right end side is the profile of the protein marker provided by the manufacturer indicating the size of the bands in kDa.
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Conclusion: E. coli strains expressing doeA are able to cleave ectoine since their cytoplasm contain DA, γ-NADA and α-NADA, compounds not found in strains lacking doeA.
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Figure 57. $^{13}$C NMR of molecules obtained as products of the alkaline hydrolysis of hydroxyectoine.
Figure 58. HPLC comparison of FMOC derivatized molecules to identify hydroxyectoine degradation compounds produced during the in vivo test of recombinant proteins. a) Hydrolyzed hydroxyectoine (four products) b) Metabolites extracted from the cytoplasm of cells expressing target proteins (legend on the chart) c) Molecules present in the media providing nutrient to cells expressing target proteins (legend on the chart). Samples from chromatograms a and b were taken 4 h after induction (Figure 6). Conclusion: E. coli strain expressing doeA is able to cleave hydroxyectoine since its cytoplasm contain metabolites (possibly HO-DA, γ-NA-HO-DA and α-NA-HO-DA) not found in strains lacking doeA. Appropriate standards were not available. Note: Defective pre-column made peaks appear with shoulders (or doublets).
Figure 59. Sequence alignment to verify the sequence inserted in vectors for deletion of **doeA** and **eutB**. a) pK18mob sacB<sub>Δ</sub>doeA sequence in black letters (region 5495-6594b 5'→3') and b) pK18mob sacB<sub>Δ</sub>eutB sequence in black letters (region 5494-6992b 5'→3'). DNA sequences experimentally determined are presented in different colors according to the primers used for their amplification: M13rev(−47) primer is in brown letters M13rev(−47) primer, in purple letters. Restriction sites are underlined and followed by a superscript letter that indicates the corresponding restriction enzyme: E=EcoRI, H=HindIII
Figure 60. Sequence alignment to verify the sequence inserted in vector used for deletion of eutC.

pK18mobsacB::EutC sequence in black letters (region 5501-7900 b 5’→3’). DNA sequences experimentally determined are presented in different colors according to the primers used for their amplification: M13rev(-48) primer are presented in brown letters, with Seq3660-F2 primer in blue letters, the complementary strand to sequence amplified with the Seq3659-F2 primer in orange letters and the complementary strand to sequence amplified with M13rev(-47) primer in purple letters. Restriction sites recognized by HindIII are underlined and followed by a superscript H (15).

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10 Affirmation

I herewith declare that I have independently written this thesis with careful consideration from the best of my scientific knowledge, used no other than the indicated sources and means, and indicated any citations from literature and personal communications.