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Feeding on compatible solutes: A substrate-induced pathway for uptake and catabolism of ectoines and its genetic control by EnuR

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Summary

Ectoine and 5-hydroxyectoine are widely synthesized microbial osmostress protectants. They are also versatile nutrients but their catabolism and the genetic regulation of the corresponding genes are incompletely understood. Using the marine bacterium *Ruegeria pomeroyi* DSS-3, we investigated the utilization of ectoines and propose a seven steps comprising catabolic route that entails an initial conversion of 5-hydroxyectoine to ectoine, the opening of the ectoine ring, and the subsequent degradation of this intermediate to L-aspartate. The catabolic genes are co-transcribed with three genes encoding a 5-hydroxyectoine/ectoine-specific TRAP transporter. A chromosomal deletion of this entire gene cluster abolishes the utilization of ectoines as carbon and nitrogen sources. The presence of ectoines in the growth medium triggers enhanced expression of the importer and catabolic operon, a process dependent on a substrate-inducible promoter that precedes this gene cluster. EnuR, a member of the MocR/GabR-type transcriptional regulators, controls the activity of this promoter and functions as a repressor. EnuR contains a covalently bound pyridoxal-5'-phosphate, and we suggest that this co-factor is critical for the substrate-mediated induction of the 5-hydroxyectoine/ectoine import and catabolic genes. Bio-informatics showed that ectoine consumers are restricted to the *Proteobacteria* and that EnuR is likely a central regulator for most ectoine/5-hydroxyectoine catabolic genes.

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Introduction

There is essentially no compound synthesized by microorganisms that cannot be catabolized, either by the producer cell itself or by other microorganisms. This is also true for compatible solutes (Welsh, 2000), which are organic osmolytes synthesized by microorganisms when they face hyper-osmotic conditions. These compounds can be accumulated to exceedingly high cellular levels to avoid dehydration of the cytoplasm and loss of turgor (Kempf and Bremer, 1998; Bremer and Krämer, 2000; Roesser and Müller, 2001). When microorganisms are suddenly exposed to hypo-osmotic souroundings, compatible solutes are rapidly released through the transient opening of mechanosensitive channels from the cell in order to curb water influx, prevent an undue raise in turgor and avoid rupture (Levina et al., 1999; Hoffmann et al., 2008; Booth, 2014). Release of these compounds into the environment will also occur when bacteria are attacked and lysed by bacteriophages or toxins or through the grazing activities of protozoa (Welsh, 2000). Compatible solutes that are present in a given habitat (Mosier et al., 2013; Warren, 2014) are valuable resources for microorganisms since they can either be scavenged via high-affinity transporters for their re-use as osmostress protectants (Bremer and Krämer, 2000), or they can be exploited as nutrients (Welsh, 2000).

The tetrahydropyrimidines ectoine and its derivative 5-hydroxyectoine are such compatible solutes (Pastor et al., 2010). They are highly effective cytoprotectants and are widely produced by members of the *Bacteria*, and a few *Archaea*, in response to osmotic stress (Widderich et al., 2014a; Widderich et al., 2015). Ectoine and 5-hydroxyectoine are not only excellent stress protectants but can also serve as valuable nutrients (Galinski and Herzog, 1990; Manzanera et al., 2002; Jebbar et al., 2005; Vargas et al., 2006; Rodriguez-Moya et al., 2010). However, in contrast to 5-hydroxyectoine/ectoine biosynthesis

(Ono et al., 1999; Stöveken et al., 2011; Höppner et al., 2014), this process is both biochemically and genetically insufficiently understood. Here we focus on the use of ectoines as sole carbon, energy, and nitrogen sources.

An inroad into the molecular biology of ectoine catabolism was made by a proteome study that identified ectoine-inducible proteins in the plant-root-associated bacterium *Sinorhizobium meliloti* (Jebbar et al., 2005). These growth-substrate-induced proteins comprised components of a binding-protein-dependent ABC transport system (EhuABCD; *ehu*: **e**ctoine-**h**ydroxyectoine **u**ptake) (Hanekop et al., 2007) and enzymes (EutABCDE; *eut*: **e**ctoine **ut**ilization) predicted as key constituents of the ectoine catabolic route (Jebbar et al., 2005). A gene encoding a transcriptional regulator of the GntR family (Rigali et al., 2002) was present in the vicinity of the 5-hydroxyectoine/ectoine uptake and catabolic genes. The expression of the *ehu-eut* gene cluster was ectoine inducible but did not respond to osmotic stress, a transcriptional profile consistent with a catabolic role of the genes under study (Jebbar et al., 2005). A gene cluster similar to that present in *S. meliloti* was identified through bioinformatics in *Ruegeria pomeroyi* DSS-3 (Lecher et al., 2009), a representative of the ecologically important *Roseobacter* clade of marine microorganisms (Wagner-Döbler and Biebl, 2006; Brinkhoff et al., 2008; Luo and Moran, 2014). However, in the *R. pomeroyi* DSS-3 genome (Moran et al., 2004; Rivers et al., 2014), the genes for the 5-hydroxyectoine/ectoine-specific EhuABCD ABC transporter (Hanekop et al., 2007) had been replaced with substrate-inducible genes encoding a member of the tripartite ATP-independent periplasmic (TRAP) transporter family, UehABC (*ueh*: **u**ptake of **e**ctoine and **h**ydroxyectoine) (Lecher et al., 2009) (Fig. 1).

When the genome sequence of *Halomonas elongata*, the salt-tolerant bacterium used for the commercial production of ectoine (Pastor et al., 2010; Kunte et al., 2014), was established, a gene cluster related to those previously implicated in ectoine degradation in *S. meliloti* and *R. pomeroyi* DSS-3 (Jebbar et al., 2005; Lecher et al., 2009) was noted but a different genetic nomenclature was used to annotate its members (Schwibbert et al., 2011). These authors made for the first time a concrete proposal for an ectoine degradation route that begins with the hydrolysis of the ectoine ring and eventually leads to the formation of L-aspartate (Fig. 1), an amino acid that readily can be fed into both anabolic and catabolic activities of bacterial cells (Lo et al., 2009). The data presented by Schwibbert et al. (2011) represent an important step forward in our understanding of the ectoine catabolic route, but important questions remain. These concern the steps that lead to the degradation of 5-hydroxyectoine, the way the 5-hydroxyectoine/ectoine catabolic genes are genetically regulated, and how widely they are distributed in the microbial world in comparison with those for 5-hydroxyectoine/ectoine biosynthesis (Widderich et al., 2014a; Widderich et al., 2015). Here we address these questions. We make a proposal for the entire 5-hydroxyectoine/ectoine catabolic route that incorporates suggestions made by Schwibbert *et al*. (2011) but also entails the biochemical steps required for the removal of the hydroxyl group from 5-hydroxyectoine to form ectoine. In contrast to the taxonomic distribution of 5-hydroxyectoine/ectoine biosynthetic genes that can be found in 14 microbial phyla (Widderich et al., 2014a; Widderich et al., 2015), predicted 5-hydroxyectoine/ectoine consumers are restricted to the phylum of the *Proteobacteria*. We demonstrate here that the transcription of the 5-hydroxyectoine/ectoine uptake and catabolic genes from *R. pomeroyi* DSS-3 is inducible by an external supply of ectoines. A regulatory gene preceding this operon encodes a protein, dubbed EnuR by us, of the MocR/GabR subfamily of GntR-type transcriptional regulators (Rigali et al., 2002; Bramucci et al., 2011; Milano et al., 2015; Suvorova and Rodionov, 2016). We show that EnuR contains a covalently bound pyridoxal-5'-phosphate (PLP) as a co-factor, and our genetic data indicate that it is a key player in the ectoine-mediated induction of the 5-hydroxyectoine/ectoine uptake and the catabolic gene cluster in *R. pomeroyi* DSS-3 and many other bacteria capable to use ectoines as nutrients.

Results

The 5-hydroxyectoine/ectoine uptake and degradation gene cluster from R. pomeroyi DSS-3

The 5-hydroxyectoine/ectoine uptake and catabolic gene cluster comprises 13 genes (Fig. 1A) and can be broken down into three functional modules: (i) The first module comprises the *uehABC* genes encoding a member of the TRAP transporter family (Mulligan et al., 2011). Its ligand-binding protein (UehA) (Fig. 1C) has been shown to bind both ectoine and 5-hydroxyectoine avidly, with K_d values in the low μM range, and the crystal structure of UehA in complex with ectoine has been reported (Lecher et al., 2009). Ectoine uptake by *R. pomeroyi* DSS-3 is substrate-inducible but not stimulated by high salinity (Lecher et al., 2009), the expected profile of a transporter that imports ectoines for metabolic purposes rather than for osmostress protection. Indeed, we found that neither ectoine nor 5-hydroxyectoine serve as an osmostress protectant for salt-stressed *R. pomeroyi* DSS-3 cells, whereas choline, glycine betaine, choline-*O*-sulfate, crotonobetaine, and carnitine provided such a cytoprotective function (Supporting Information Fig. S1). (ii) The second module comprises the *eutABC* genes (Fig. 1A), and we suggest (see below) that these three genes encode enzymes required for the removal of the hydroxyl-group from

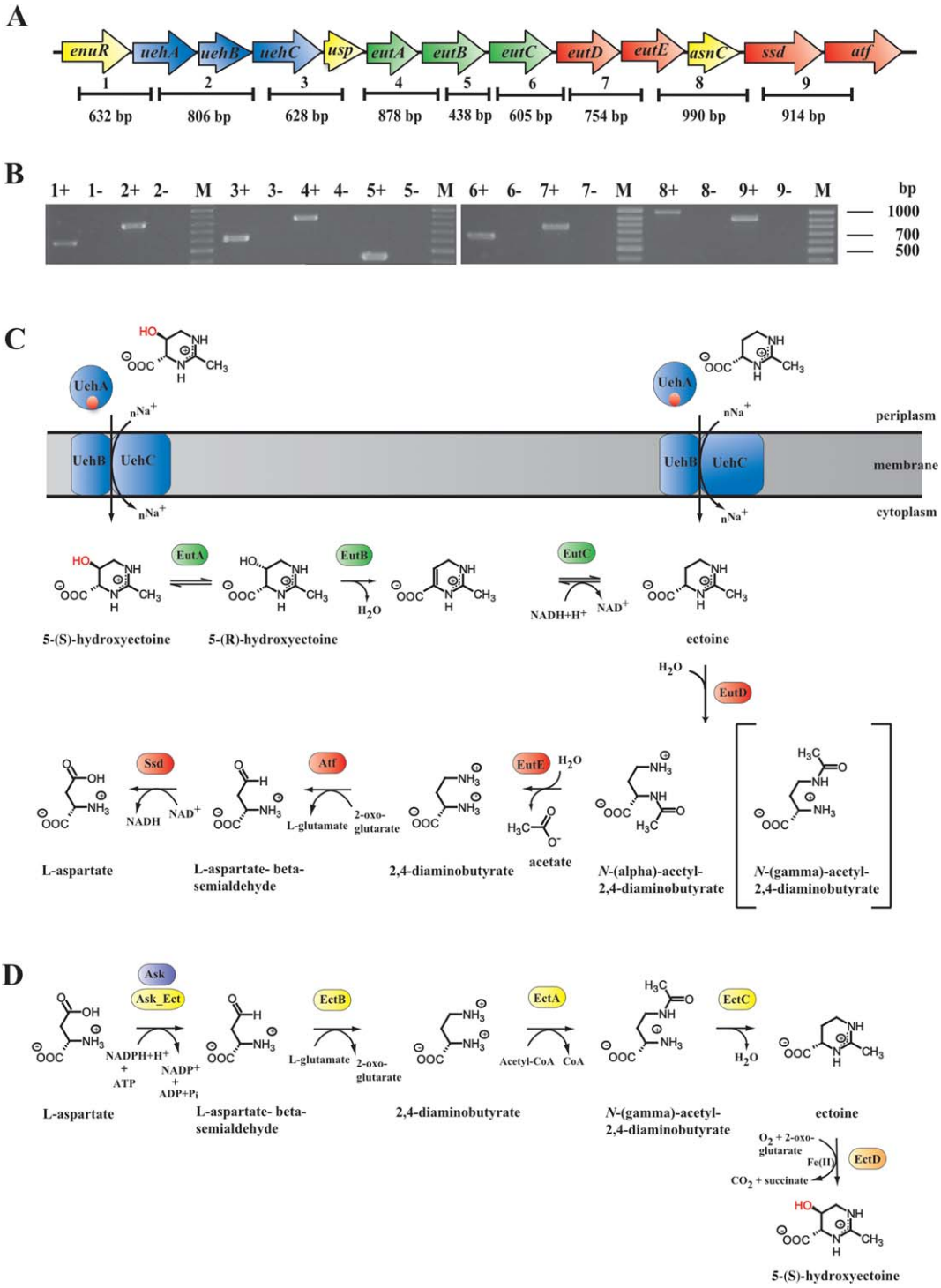

Fig. 1. Genetic organization of the 5-hydroxyctoine/ectoine uptake and utilization gene cluster and a proposal for the catabolism of these compatible solutes. (A) Genetic map of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster in the genome of *R. pomeroyi* DSS-3. The predicted length and position of the different RT-PCR-amplified DNA fragments are indicated. (B) Analysis of the DNA fragments generated through RT-PCR by agarose gel electrophoresis. The '+' symbol marks those samples that were incubated with reverse transcriptase; the symbol '-' denotes samples prepared without reverse transcriptase to ensure that the detected PCR products do not result from DNA contaminations in the used RNA sample. (C) Proposal for the enzymatic pathway leading to the catabolism of 5-hydroxyectoine and ectoine to L-aspartate. Based on database and literature searches, gene products of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster were annotated and assigned a function in the 5-hydroxyectoine/ectoine catabolic pathway. (D) Enzymatic steps catalysed by the ectoine and 5-hydroxyectoine biosynthetic proteins (EctABCD).

5-hydroxyectoine to yield ectoine (Fig. 1C). (iii) The third module is formed by the *eutDE-ssd-atf* genes (Fig. 1A) that encode enzymes for the hydrolysis of the ectoine ring (EutD) and the further catabolism of the formed *N*-α-acetyl-L−2,4-diaminobutyric acid (*N*-α-ADABA) to L-aspartate (Fig. 1C). This latter module is identical to the one previously proposed for ectoine degradation by *H. elongata* (Schwibbert et al., 2011). It should be noted in this context that *H. elongata* can both synthesize and catabolize 5-hydroxyectoine/ectoine (Schwibbert et al., 2011), and contains an independent gene cluster for 5-hydroxyectoine/ectoine biosynthesis (Louis and Galinski, 1997; Bursy et al., 2007). These biosynthetic genes are not present in *R. pomeroyi* DSS-3 (Moran et al., 2004; Rivers et al., 2014).

Three genes of the 5-hydroxyectoine/ectoine uptake and catabolic gene cluster (Fig. 1A) encode putative regulatory proteins. The gene preceding the *uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster encodes a member of the GntR superfamily of transcriptional regulators (Rigali et al., 2002) and belongs to the MocR/GabR-type sub-group (Bramucci et al., 2011; Milano et al., 2015; Suvorova and Rodionov, 2016). We will refer in the following to this regulatory gene as *enuR* (**e**ctoine **n**utrient **u**tilization **r**egulator). The structurally and functionally best-characterized member of the MocR family is GabR from *Bacillus subtilis*, a regulatory protein involved in γ-aminobutyric acid metabolism, which is an important nitrogen and carbon source in many bacteria (Belitsky and Sonenshein, 2002; Belitsky, 2004; Edayathumangalam et al., 2013).

A gene (*usp*) encoding for a member of the universal stress protein family (Tkaczuk et al., 2013) follows the *uehABC* genes (Fig. 1A). Usp proteins are encoded in many operons for TRAP-transport systems (Mulligan et al., 2011), including that for the osmotically (but not substrate) inducible TeaABC 5-hydroxyectoine/ectoine transporter of *H. elongata* (Grammann et al., 2002; Kuhlmann et al., 2008). The Usp protein (TeaD) associated with the TeaABC system from *H. elongata* has been crystalized and seems to modulate TeaABC-mediated ectoine uptake activity but its mode of action is unresolved (Schweikhard et al., 2010).

The *asnC* gene embedded in the 5-hydroxyectoine/ectoine uptake and catabolic gene cluster of *R. pomeroyi* DSS-3 (Fig. 1A) encodes a regulatory protein belonging to the "feast and famine" family of transcriptional regulators (Deng et al., 2011). These types of regulatory proteins often form octamers wrapping the target DNA around them and their DNA-binding properties are dictated by small molecules, mostly amino acids (e.g., L-Leucine, L-Glutamine, L-Alanine, L-Tyrosine, L-Tryptophane, or L-Aspartate) (Kumarevel et al., 2008; Dey et al., 2016).

Transcriptional organization of the 5-hydroxyectoine/ectoine uptake and catabolic gene cluster

To test for a possible co-transcription of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster (11A), we carried out a reverse transcription polymerase chain reaction (RT-PCR) analysis. Since previous experiments had hinted that the transcription of this cluster is substrate inducible (Lecher et al., 2009), we used for this experiment total RNA preparations from *R. pomeroyi* DSS-3 cells that had been grown in the presence of ectoine. Using nine pairs of DNA primers that span all intergenic regions of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster (Fig. 1A), we recorded formation of all predicted amplicons dependent on the presence of reverse transcriptase in the assay buffer. Therefore, these 13 genes are transcribed as an operon (Fig. 1B), but the performed RT-PCR analysis does not allow conclusions about possible sub-transcriptional units that might be present in this very large gene cluster of 13,503 bp (Fig. 1A).

Substrate-induced transcription of 5-hydroxyectoine and ectoine uptake and catabolism genes

Expression of genes involved in the uptake and catabolism of a given compound by microorganisms is often induced by the presence of this compound in the growth medium. Indeed, the UehABC-mediated import of radiolabeled ectoine in *R. pomeroyi* DSS-3 is stimulated in cells that are grown in the presence of ectoine (Lecher et al., 2009). We therefore assessed a potential increase in the level of transcription of the *R. pomeroyi* DSS-3 *uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster in response to the presence or absence of ectoine or 5-hydroxyectoine in the growth medium by dot blot hybridization analyses. To this end, we isolated total RNA from *R. pomeroyi* DSS-3 cells grown either with glucose, ectoine, or 5-hydroxyectoine as the carbon source and probed for the level of transcription of representative genes either in the front (*enuR*, *uehA*), or the end segment (*ssd*, *atf*) of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster. These data showed that the level of transcription of the *enuR* regulatory gene was low, but independent of the available substrates, whereas the transcripts of *uehA, ssd* and *atf* were strongly up-regulated in cells grown on either ectoine or 5-hydroxyectoine in the growth medium (Supporting Information Fig. S2). As a control, we investigated the transcriptional profile of *dddW*, a gene involved in the catabolism of the ecologically abundant compatible solute and important sulfur source in marine ecosystems, dimethylsulfoniopropionate (DMSP) (Todd et al., 2012a); its transcriptional level was not up-regulated in response to ectoine or 5-hydroxyectoine availability (Supporting Information Fig. S2).

Deletion of the enuR-uehABC-usp-eutABCDE-asnC-ssd-atf gene cluster abolishes 5-hydroxyectoine and ectoine catabolism

To prove that the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster is required for the utilization of ectoine and 5-hydroxyectoine as nutrients, we constructed a chromosomal deletion of the entire operon and compared the growth properties of the resulting mutant (strain ASR6) with those of its parent *R. pomeroyi* strain J470 (Supporting Information Table S1). Both ectoines were effectively used by the wild-type strain as sole carbon, nitrogen, and energy sources (Fig. 2A). As foreseen, the mutant derivative (ASR6) lost the ability to use ectoines as nutrients (Fig. 2A). Hence, the predicted gene products of the *uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster do not only resemble those of the ectoine catabolic systems of *S. meliloti* and *H. elongata* (Jebbar et al., 2005; Schwibbert et al., 2011), but the deletion of this gene cluster in *R. pomeroyi* also results in the expected growth phenotype as well (Fig. 2A). Furthermore, a mutant (strain ASR12) possessing intact genes for the UehABC 5-hydroxyectoine/ectoine TRAP-type import system (Lecher et al., 2009) but defective in the *eutABCDE-asnC-ssd-atf* genes was unable to catabolize either 5-hydroxyectoine or ectoine (Fig. 2B). Particularly noteworthy in this context is that the deletion strains ASR6 and ASR12 had not only lost the ability to utilize ectoine but were defective in 5-hydroxyectoine utilization as well (Fig. 2A), a growth profile that had not been evaluated in previous studies with *S. meliloti* and *H. elongata* (Jebbar et al., 2005; Schwibbert et al., 2011). The inability of strains ASR6 and ASR12 to exploit ectoines as sole carbon, nitrogen, and energy sources was not caused by a general growth defect of these mutants in a chemically defined medium as they grew with glucose as the carbon source and ammonium as the nitrogen source (Supporting Information Fig. S3). Interestingly, the use of ectoine as sole carbon and nitrogen source by *R. pomeroyi* consistently yielded a higher biomass in comparison with the use of 5-hydroxyectoine (Fig. 2A).

A proposal for the conversion of 5-hydroxyectoine to ectoine by the EutABC enzymes

We used database and literature searches to functionally annotate the probable roles of the 5-hydroxyectoine and ectoine catabolic enzymes EutA-E, Atf and Ssd. The proposed pathway starts with the conversion of 5-hydroxyectoine to ectoine by the products of the *eutABC* genes. Degradation of 5-hydroxyectoine is proposed to be initiated via steric inversion of its hydroxyl group by the EutA enzyme, converting the native 5-(*S*)-hydroxyectoine (Inbar et al., 1993) to the 5-(*R*)-enantiomer to fit the stereochemical requirements of the next enzyme, EutB (Fig. 1C). EutA belongs to a protein family of racemases (pfam01177), which also includes MurI, a glutamate racemase synthesizing D-glutamate for bacterial peptidoglycan synthesis (Spies et al., 2009). MurI contains two conserved catalytically important cysteines in the active site (Puig et al., 2009), and these two Cys residues are also present in the same relative position of the EutA protein (Cys-94 and Cys-204). The next step appears to be a water elimination catalyzed by the EutB enzyme (Fig. 1C), which is predicted to be a PLP-containing enzyme similar to threonine dehydratases (superfamily cl00342) (Gallagher et al., 2004). In comparison with this latter enzyme, the EutB protein contains all necessary consensus features involved in catalysis including Lys-54, to which the PLP molecule is predicted to be covalently attached (Gallagher et al., 1998). As the latter enzymes are specific for eliminating the (*R*)-hydroxyl group of threonine, it may be envisioned that EutB only converts the analogous 5-(*R*)-enantiomer of hydroxyectoine, which does not occur naturally (Inbar et al., 1993), and therefore needs to be generated by EutA or another racemase. The predicted product is 2-methyl-4,5-dihydropyrimidine-6-carboxylate, which is subsequently proposed to be reduced to ectoine by the EutC enzyme (Fig. 1C). EutC has been annotated in the genome sequence of *R. pomeroyi* (Moran et al., 2004) as an ornithine cyclodeaminase, an enzyme catalyzing the reversible conversion of ornithine to proline and ammonia via a tightly bound NAD cofactor in an internal redox loop (Goodman et al., 2004). In contrast to this reaction, we propose EutC to catalyze a simple NADH-dependent reduction, acting as a reversible "ectoine dehydrogenase". This proposed function of EutC is further substantiated by the presence of enzymes within the ornithine cyclodeaminase family (pfam02423) that act as normal dehydrogenases without performing redox-loop reactions, e.g. the archaeal alanine dehydrogenase (Gallagher et al., 2004; Schröder et al., 2004). A detailed description of the three proposed steps for the conversion of 5-hydroxyectoine to ectoine can be found in Supporting Information Figure S4.

Our proposal that the EutABC proteins catalyze the conversion of 5-hydroxyectoine to ectoine (Fig. 1C) predicts that the disruption of the corresponding structural genes for these enzymes should abolish the use of 5-hydroxyectoine as a nutrient, whereas growth on ectoine should still be possible. We tested this hypothesis by constructing a complete chromosomal deletion of the *eutABC* genes and replaced them with a non-polar gentamycin (Gm) resistance cassette derived from plasmid p34S_GM (Dennis and Zylstra, 1998; Lidbury et al., 2014). In full agreement with our proposal, we found that the resulting mutant strain ASR11 [Δ(*eutABC*::Gm^r)] still grew on ectoine but lost the ability to use 5-hydroxyectoine as sole carbon and nitrogen source (Fig. 2C).

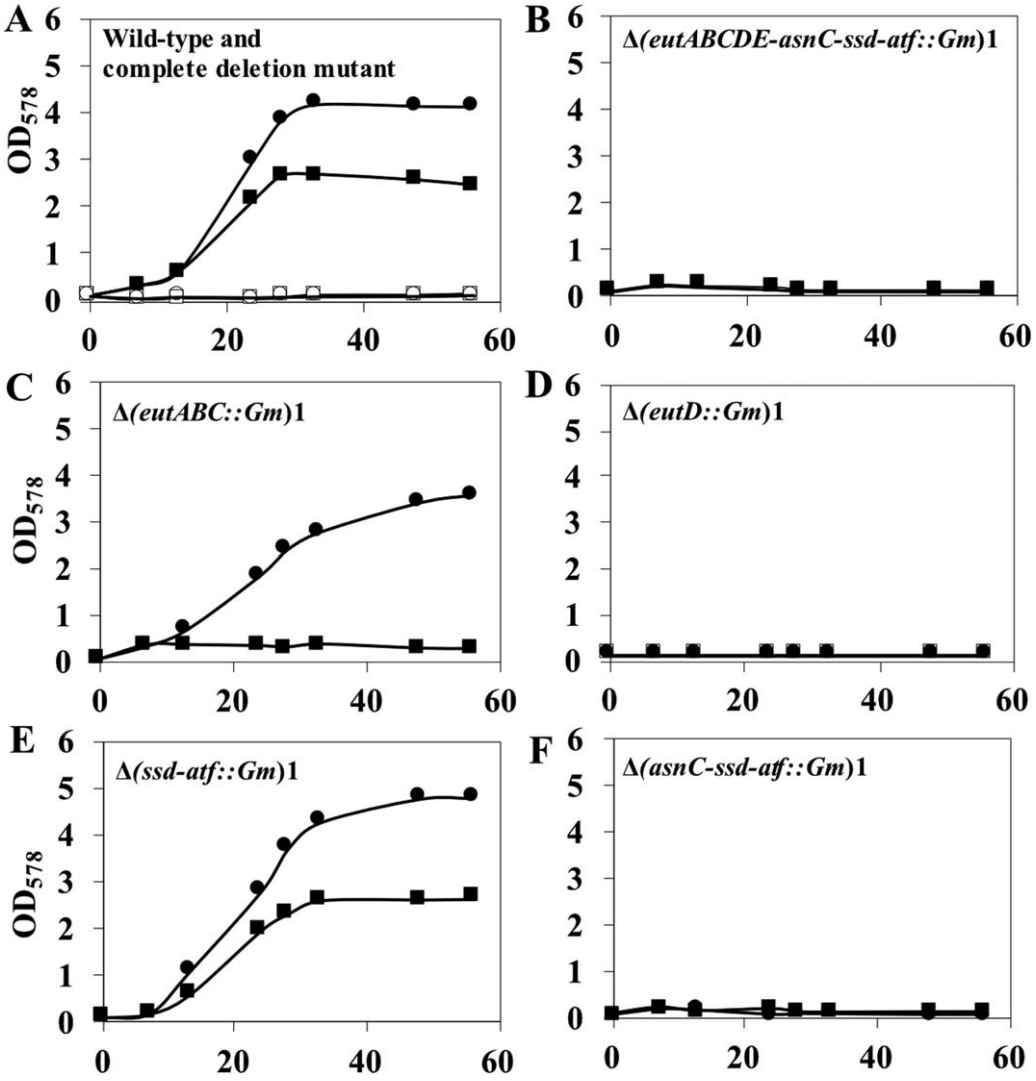

Fig. 2. Growth curves of cultures of the rifampicin-resistant *R. pomeroyi* DSS-3 derivative, strain *R. pomeroyi* J470, and its mutant derivatives defective in various regulatory and ectoine catabolic genes in basal minimal media containing either ectoine (circles) or 5-hydroxyectoine (squares) as a sole carbon and nitrogen sources, are presented; ectoine and 5-hydroxyectoine were present in these cultures at a concentration of 28 mM. (A) Growth of the wild-type *R. pomeroyi* J470 strain (solid symbols) and its mutant derivative *R. pomeryi* strain ASR6 Δ(*enuR-uehABC-usp-eutABCDE-asnC-ssd-atf::Gm*)1 (open symbols) (B) Growth of the *R. pomeryi* strain ASR6 Δ(*eutABCDE-asnC-ssd-atf::Gm*)1 (C) Growth of the *R. pomeryi* strain ASR11 Δ(*eutABC::Gm*)1 (D) Growth of the *R. pomeryi* strain ASR8 Δ(*eutDf::Gm*)1 (E) Growth of the *R. pomeryi* strain ASR14 Δ(*ssd-atf::Gm*)1 (F) Growth of the *R. pomeryi* strain ASR12 Δ(*asnC-ssd-atf::Gm*)1.

Further pathway of ectoine degradation

The involvement of the products of the further genes in the *eut* gene cluster (Fig. 1C) in ectoine degradation has been identified previously by genetic experiments and partial overexpression of the respective genes (Jebbar et al., 2005; Schwibbert et al., 2011). The *eutD* gene codes for an ectoine hydrolase, and the heterologous expression of the corresponding gene (*doeA*) from *H. elongata* in *Escherichia coli* leads to the formation of both *N*-γ-acetyl-L−2,4-diaminobutyric acid (*N*-γ-ADABA) and its isomer *N*-α-ADABA (Schwibbert et al., 2011). *N*-γ-ADABA is the natural substrate for the ectoine synthase (EctC) (Ono et al., 1999; Widderich et al., 2016) which catalyzes the last step in ectoine biosynthesis (Peters et al., 1990) (Fig. 1D). *N*-α-ADABA, on the other hand, is funneled in the last part of the ectoine catabolic route (Schwibbert et al., 2011). Through its ring-opening enzyme activity, the EutD/DoeA protein performs a key step in ectoine degradation (Fig. 1C). EutD is closely related to the M24 family of peptidases (pfam00557), which also include amino-peptidases and prolidase (Rawlings and Barrett, 2013). The next step of *N*-α-ADABA degradation is catalyzed by the *eutE* gene

product, as suggested by the observed accumulation of both *N*-α-ADABA and *N*-γ-ADABA, a *H. elongata doeB* (*eutE*) deletion mutant (Schwibbert et al., 2011). Because *H. elongata* wild-type cells only accumulated the *N*-γ-ADABA isomer, the EutE/DoeB protein appears to function as a specific *N*-α-ADABA deacetylase, generating acetate and 2,4-diaminobutyrate as products (Schwibbert et al., 2011). EutE/DoeB is related to the M14 family of zinc-dependent carboxypeptidases (pfam00246), which also includes succinylglutamate desuccinylases/aspartocylases and γ-glutamyl-diaminopimelate peptidases involved in bacterial cell wall turnover (Rawlings and Barrett, 2013). Whereas the released acetate is degraded via the TCA cycle, 2,4-diaminobutyrate is further converted to aspartate semialdehyde by transamination at C4, which could be catalyzed by the Atf/DoeD enzyme (Fig. 1C). This protein belongs to the acetyl ornithine aminotransferase family of PLP-dependent aminotransferases (Christen and Mehta, 2001). Finally, the formed aspartate semialdehyde is apparently oxidized to L-aspartate by the *ssd/doeC* gene product, which is highly similar to known succinate semialdehyde dehydrogenases (Jebbar et al., 2005; Schwibbert et al., 2011). A *ssd* deletion mutant in *H. elongata* was able to utilize ectoine as nitrogen-source (Schwibbert et al., 2011), establishing that the reaction of this enzyme can only occur after at least one of the two nitrogen atoms of ectoine has been channeled into the anabolic metabolism (Fig. 1C).

The *eutD*-encoded ectoine hydrolase assumes a special position in our proposed catabolic pathway since the use of both 5-hydroxyectoine and ectoine as nutrients is dependent on the function of this enzyme (Fig. 1C). Indeed, the [Δ(*eutD*::Gm^r)] mutant strain (ASR8) of *R. pomeroyi* was defective in the utilization of either of these compounds (Fig. 2D), a growth phenotype that has also been observed for the corresponding *doeA* mutant of *H. elongata* (Schwibbert et al., 2011). The Atf and Ssd enzymes from the 5-hydroxyectoine/ectoine catabolic route (Fig. 1C) have counterparts in the general amino acid metabolism of microorganisms. Therefore, our finding that their loss in strain ASR14 [Δ(*ssd-atf*::Gm^r)] has no consequences for 5-hydroxyectoine/ectoine catabolism (Fig. 2E) is thus not particularly surprising and is consistent with data reported for *H. elongata* (Schwibbert et al., 2011). However, their deletion in concert with the *asnC* regulatory gene in the *R. pomeroyi* strain ASR10 [Δ(*asnC-ssd-atf*::Gm^r)] caused failure of 5-hydroxyectoine/ectoine utilization (Fig. 2E). This observation suggests an important regulatory function of the "feast and famine"-type AsnC transcriptional regulator (Kumarevel et al., 2008; Deng et al., 2011; Dey et al., 2016) for the expression of the 5-hydroxyectoine/ectoine uptake and catabolic gene cluster of *R. pomeroyi*.

Analysis of the enuR and uehA transcriptional profile by reporter gene fusion studies

The dot blot hybridization analysis presented in Supporting Information Figure S2 revealed that the expression of the *enuR* and *uehA* genes responds differently to the presence of 5-hydroxyectoine and ectoine in the growth medium, despite the fact that they are co-transcribed (Fig. 1B) Expression of *uehA* is strongly inducible by 5-hydroxyectoine and ectoine, whereas expression of *enuR* appears to be constitutive at a low basal level (Supporting Information Fig. S2). These data indicate that *enuR* and *uehA* are expressed from genetically differently regulated promoters.

To investigate this in greater detail, we constructed a set of *lacZ* transcriptional reporter fusions (Fig. 3A) using plasmid pBIO1878 (Todd et al., 2012a) as vector. We introduced the corresponding plasmids into the *R. pomeroyi* wild-type strain J470 and asked whether the expression of the *lacZ* reporter gene was influenced by the presence of ectoine in the growth medium. The *enuR-lacZ* fusion (present on plasmid pBAS19) was constitutively expressed at a low basal level, whereas the transcription of the *uehA-lacZ* fusion (present on plasmid pBAS21) was substrate inducible (Fig. 3A). Hence, an ectoine-inducible internal promoter must be present either in the 3' region of the *enuR* gene or in the *enuR-uehA* intergenic region (Fig. 3A). This substrate-inducible promoter is likely responsible for the transcription of the entire *uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster (Fig. 1A and B).

The co-transcription of *enuR* with *uehA* from the constitutive *enuR* promoter (Fig. 1B) suggests a role of the EnuR protein in controlling the expression of the *uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster. Bioinformatics showed that EnuR is a GntR-type regulator (Rigali et al., 2002) and belongs to the MocR/GabR-sub-group of transcription factors, regulators that often contain a covalently attached pyridoxal-5'-phosphate as a cofactor (Bramucci et al., 2011; Suvorova and Rodionov, 2016). The few functionally characterized representatives of this large family of transcription factors are either activators or autorepressors and control both biosynthetic and catabolic genes (Bramucci et al., 2011; Suvorova and Rodionov, 2016). To assess the function of EnuR, we constructed a chromosomal deletion of its structural gene and used the resulting strain ASR7 [Δ(*enuR*::Gm^r)] for both growth assays and reporter gene analysis. Loss of the EnuR regulator resulted in a constitutive expression of the substrate-inducible *uehA* promoter but did not affect the *enuR* promoter (Fig. 3A). Hence, EnuR is involved in the regulation of the *uehABC-usp-eutABCDE-asnC-ssd-atf* operon, but in contrast to other studied MocR/GabR-type regulators (Bramucci et al., 2011; Suvorova and Rodionov, 2016), EnuR serves as a repressor. Consistent with this

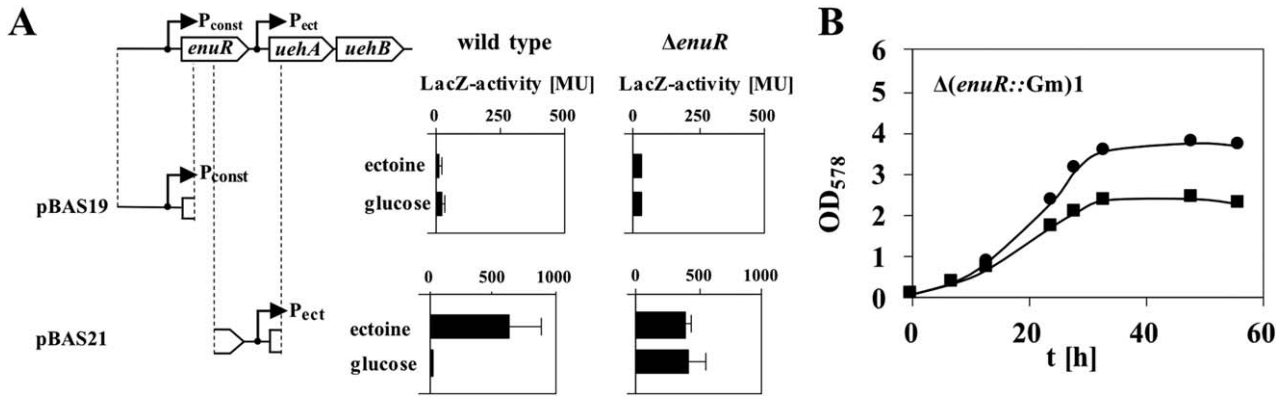

Fig. 3. Influence of the EnuR protein on putative promoter regions located upstream of *enuR* and *uehA*, respectively (A) The *lacZ* reporter gene plasmids pBAS19 (containing a DNA-region located upstream of the *enuR* gene) and pBAS21 (containing a DNA-region upstream of the *uehA* gene) were conjugated into *R. pomeroyi* J470 (wild-type) or its Δ(*enuR*) derivative strain ASR7. Cultures were grown in minimal medium containing either glucose (28 mM) as the carbon source and NH_4Cl (200 mM) as the nitrogen source, or ectoine as a sole carbon and nitrogen source (28 mM). All cell samples were harvested for LacZ enzyme activity measurements at the mid-exponential growth phase (OD_{578} of about 1.0) (B) growth of the Δ(*enuR*) mutant strain *R. pomeroyi* ASR7 in minimal medium in the presence of either ectoine (circles) or 5-hydroxyectoine (squares) as a sole carbon and nitrogen source.

finding is the growth phenotype of the *enuR* strain ASR7; it is able to use both ectoine and 5-hydroxyectoine as nutrients (Fig. 3B).

Biochemical characterization of the EnuR protein

The EnuR protein from *R. pomeroyi* DSS-3 possesses 464 amino acids. In structure-based homology modeling attempts via the SWISS-Modell server (Biasini et al., 2014), the crystal structure of the *B. subtilis* GabR protein (PDB accession code 4N0B) (Edayathumangalam et al., 2013) was automatically chosen as the template. According to this *in silico* model, the EnuR protein possess a DNA-reading head that spans the N-terminal amino acids 1 to 75, followed by a 25 amino acid linker sequence attached to a 364 amino acid C-terminal aminotransferase-I domain (Supporting Information Fig. S5A). The aminotransferase-I domain of EnuR is predicted to contain a covalently attached PLP molecule (Milano et al., 2015; Suvorova and Rodionov, 2016) that should be bound to the structurally conserved Lys-302 residue (Supporting Information Fig. S5B).

To study EnuR from *R. pomeroyi* DSS-3 biochemically, we obtained a codon-optimized *enuR* gene for its heterologous expression in *E. coli* in the form of an EnuR-Strep tag II hybrid protein. We were able to purify this protein by affinity chromatography on a Strep-TactinSuperflow matrix to near homogeneity (Supporting Information Fig. S6A). EnuR preparations were largely free of protein aggregates and the analysis of its quaternary assembly by size-exclusion chromatography (SEC) indicated that EnuR forms homodimers in solution (Supporting Information Fig. S6A). It eluted from the size-exclusion column as a 102.73-kDa species; the calculated molecular mass of the monomer of the EnuR-Strep tag II protein is 51.97 kDa.

The heterologously expressed and purified EnuR-Strep tag II hybrid protein had a distinct yellow color (Fig. 4B), indicative of the presence of a chromophore. This chromophore is predicted to be PLP, as expected from studies assessing the aminotransferase-I domain of MocR/GabR-type regulators (Edayathumangalam et al., 2013; Okuda et al., 2015a; Okuda et al., 2015b; Suvorova and Rodionov, 2016). Indeed, EnuR protein preparations had a UV–Vis spectrum characteristic for PLP-containing proteins (Olmo et al., 2002), with absorption maxima at 330 and 420 nm (Fig. 4C). To prove that the yellow color of EnuR protein preparations resulted from the covalent attachment of a PLP molecule to Lys-302 (Supporting Information Fig. S5B), we changed the corresponding codon for Lys-302 via site-directed mutagenesis to a codon for a His residue. When the resulting EnuR(K/H) mutant protein (EnuR*) was purified, it had lost the yellow color exhibited by the wild-type EnuR protein (Fig. 4B) and the characteristic UV–Vis spectrum was altered in a fashion that indicated the loss of the PLP co-factor (Fig. 4C). The Lys-302 to His mutation did not affect the overall fold of the EnuR* protein, since it was still able to form dimers in solution (Supporting Information Fig. S6B). Taken together, both the *in silico* modeling (Supporting Information Fig. S5) and our biochemical studies (Fig. 4) demonstrate that EnuR belongs to the MocR/GabR family of transcriptional regulators and contains, like other MocR/GabR-type regulators, a covalently attached PLP molecule (Edayathumangalam et al., 2013; Milano et al., 2015; Okuda et al., 2015a; Okuda et al., 2015b; Suvorova and Rodionov, 2016).

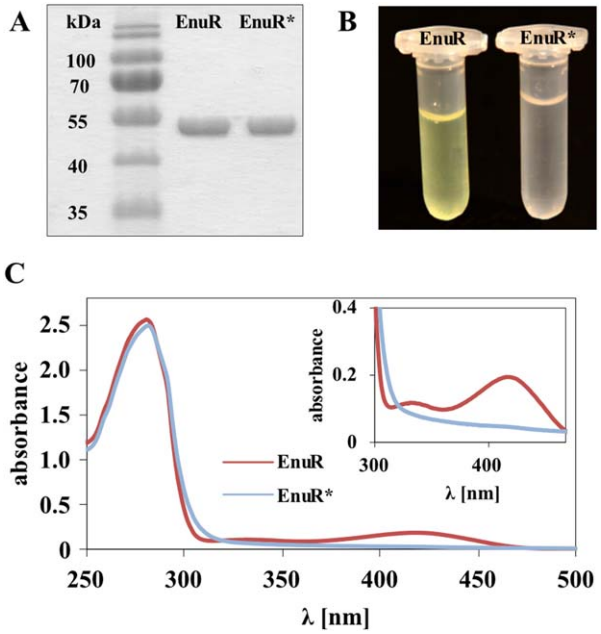

Fig. 4. Recombinant overproduction of EnuR and its Lys302/His mutant (EnuR*), and assessment of spectral properties of these regulatory proteins. (A) EnuR and EnuR* were overproduced in *E. coli* and purified using *Strep*-tag-II affinity chromatography. 2 μg of each protein was applied onto a 12% SDS-PAGE. The PageRuler Prestained Protein Ladder (Therno Scientific, Schwerte, Germany) was used as the marker. (B) Solutions (3 mg mL^{-1}) of the purified EnuR (yellow) and EnuR* (colorless) proteins. (C) Absorption spectra of 20 μM EnuR and EnuR*, respectively, were recorded at 25°C in 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The absorption maxima at 330 and 420 nm, characteristic of proteins containing PLP as a covalently attached co-factor (red line), are lost in the EnuR* (Lys302/His) mutant, which is defective in PLP binding.

Genome context of the 5-hydroxyectoine/ectoine catabolic gene clusters

The ring-opening ectoine hydrolase EutD is the key enzyme for the 5-hydroxyectoine/ectoine catabolic route (Fig. 1C). It is thus reasonable to assume that it is well conserved among the enzymes involved in 5-hydroxyectoine/ectoine catabolism. EutD therefore seemed a good representative for assessing this physiological trait through the mining of microbial genome sequences. For this purpose, we searched the database of the Joint Genome Institute (JGI) of the US Department of Energy (http://jgi.doe.gov/) (Nordberg et al., 2013) for EutD-related proteins using the amino acid sequence of the *R. pomeroyi* DSS-3 EutD protein (Moran et al., 2004) as the search query. 539 EutD orthologes were found in genome sequences of 32 523 bacterial and 654 archaeal inspected strains. We regarded in this database search only proteins as EutD-related when the corresponding genes co-localized with other genes from the *eut* gene cluster (Jebbar et al., 2005; Schwibbert et al., 2011).

When we inspected the genome context of the *eutD* gene with the aid of the neighborhood tool provided by the JGI web-site, we found that the genetic arrangement of the 5-hydroxyectoine/ectoine catabolic gene clusters was highly variable, as already noted previously in an inspection of a considerable smaller number of genome sequences (Schwibbert et al., 2011). Not only was the genetic arrangement of these genes flexible, but also the number of genes in these clusters varied, as documented for a number of examples in Figure 5. Our bioinformatics analysis suggests that among the 539 predicted ectoine-catabolizing microorganisms, 71 lack the *eutABC* genes (Supporting Information Fig. S7), which might indicate that they cannot catabolize 5-hydroxyectoine, or that they accomplish the conversion of 5-hydroxyectoine to ectoine via a route different from the one proposed here (Fig. 1C).

We consider that a minimal module for the degradation of ectoine will consist of the EutD ectoine hydrolase and the EutE deacetylase that is proposed to cleave the *N*-α-ADABA formed by EutD to acetate and 2,4-diamino butyrate (Schwibbert et al., 2011) (Fig. 1C), a common intermediate in amino acid metabolism. Indeed, we have found that the Atf and Ssd enzymes that jointly metabolize 2,4-diamino butyrate are not essential for ectoine catabolism in *R. pomeroyi* (Fig. 2E) and may be replaced by enzymes of another catabolic pathway. We noted, that 25 of the 539 predicted ectoine consumers, *eutE* was replaced by a homolog of *argE*, the gene for *N*-acetylornitine deacetylase involved in arginine biosynthesis which belongs to the M20 peptidae family (pfam01546) (Boyen et al., 1992; Tao et al., 2012). The EutE and ArgE proteins are both amidohydrolases removing acetate residues from amidic bonds and thereby may provide equivalent enzymatic function to the 5-hydroxyectoine/ectoine catabolic route (Fig. 1C).

By searching the genome sequences of the 539 predicted ectoine consumers for the presence of the *ectABC*(*D*) 5-hydroxyectoine/ectoine biosynthetic genes (Widderich et al., 2014a; Widderich et al., 2015), we found that 100 bacteria are also putative ectoine producers. 33 of these predicted ectoine producers also possess the *ectD* ectoine hydroxylase gene and hence are thus predicted to synthesize 5-hydroxyectoine as well (Supporting Information Fig. S7). Overall, a noticeable fraction (about 18.5%) of the microbial ectoine degraders are also ectoine producers. The most prominent member of this group is *H. elongata*, the halo-tolerant microorganism used as natural cell factory for the industrial scale production of ectoines (Pastor et al., 2010; Schwibbert et al., 2011; Kunte et al., 2014).

Before microbial cells can catabolize 5-hydoxyectoine and ectoine, they must be imported. Two different types of ligand-binding-protein-dependent transport systems have previously been implicated in the import of 5-

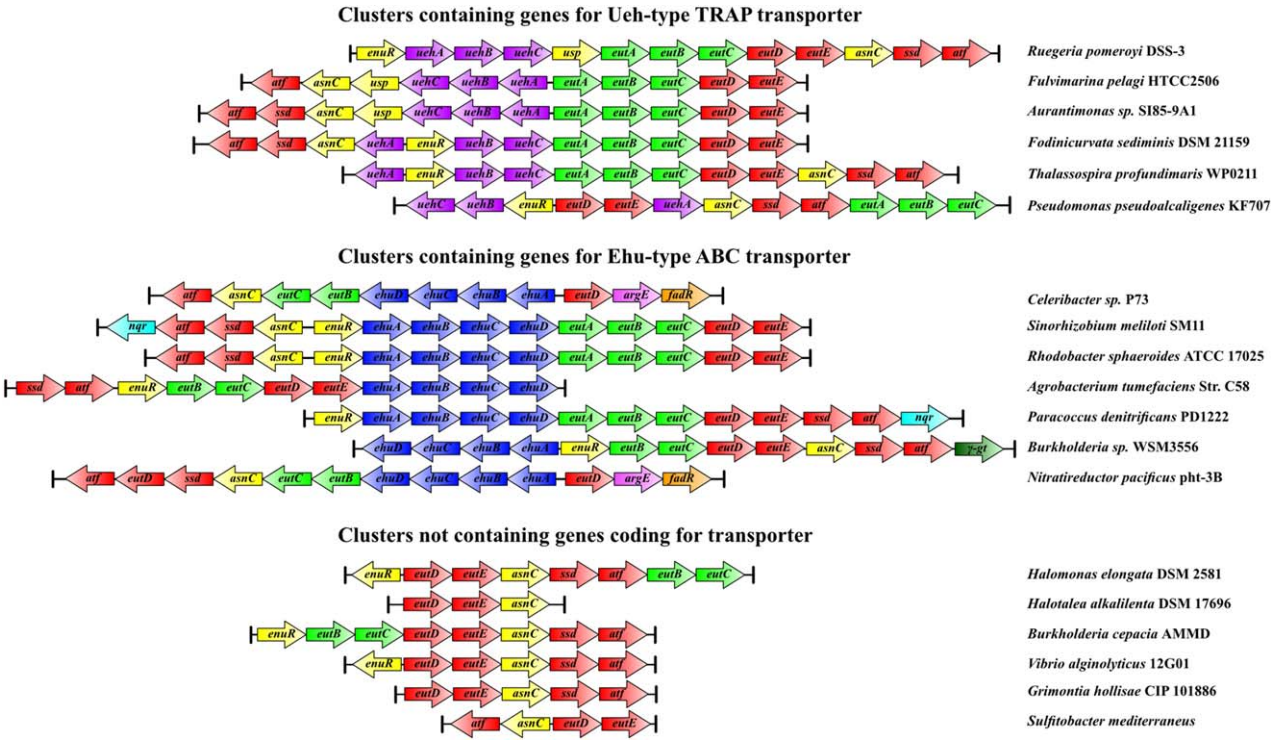

Fig. 5. Examples of the genetic organization of the 5-hydroxyectoine/ectoine catabolic gene clusters. Groups were arranged depending on the presence of the genes for the Ehu ABC and Ueh TRAP transporters, or the absence of a transport system in the direct vicinity of the 5-hydroxyectoine/ectoine catabolic gene clusters.

hydoxyectoine and ectoine when they are used as nutrients (Jebbar et al., 2005; Hanekop et al., 2007; Lecher et al., 2009): (i) EhuABCD, a member of the ATP-binding cassette (ABC) type transporters (Davidson et al., 2008), and UehABC, a member the TRAP transporter family (Mulligan et al., 2011). Their extracellular solute binding proteins have been crystalized in complex with their substrates, thereby revealing the molecular determinants for high-affinity binding of 5-hydroxyectoine and ectoine (Hanekop et al., 2007; Lecher et al., 2009).

The vast majority of the putative 539 5-hydroxyectoine/ectoine degraders possess genes for these types of transporters in the immediate vicinity of the catabolic genes; 370 had a EhuABCD-type ABC transporter and 48 possessed a UehABC-type TRAP transporter. In amino acid alignments of the periplasmic ligand-binding proteins, the ectoine- and 5-hydroxyectoine-contacting residues are functionally conserved. Exemplary amino acid alignments of the EhuB and UehA substrate-binding proteins are shown in Supporting Information Figures S8 and S9, respectively.

In the remaining 122 5-hydroxyectoine/ectoine degraders, no genes encoding for a potential transport system were associated directly with the catabolic gene cluster. However, a sub-group of these (23 representatives) possessed genes for a TeaABC-type TRAP transporter (Grammann et al., 2002) somewhere else in their genomes. The best understood TeaABC transporter is that of *H. elongata*, which serves as an osmotically inducible uptake system for ectoines when they are used as osmostress protectants and as a recycling system for newly synthesized ectoine leaking from the producer cells (Grammann et al., 2002). Although the Tea and Ueh transporters serve different physiological functions and the transcription of their structural genes is induced by different environmental cues (Grammann et al., 2002; Jebbar et al., 2005), the crystal structures of their ligand-binding proteins (UehA and TeaA) and the architecture of the ligand-binding sites (Supporting Information Fig. S9 and S10) of these solute receptors are superimposable (Kuhlmann et al., 2008; Lecher et al., 2009). Strikingly, all microorganisms possessing TeaABC-type TRAP transporters are also predicted 5-hydroxyectoine/ectoine producers (Supporting Information Fig. S7). The vast majority of those microorganisms possessing TeaABC-type transporters live in either marine or high salinity habitats (Supporting Information Fig. S7), environmental conditions that will not only trigger enhanced expression of the *teaABC* genes (Grammann et al., 2002), but Na^+ ions gradients are also used to energize substrate translocation via the TeaABC transporter (Mulligan et al., 2011). One wonders whether TeaABC-type TRAP-transporters might not only serve for

the import of ectoines when they are used as osmostress protectants but also when they are exploited as nutrients.

From the search of 5-hydroxyectoine/ectoine transporters among the 539 predicted 5-hydroxyectoine/ectoine producers, 97 did not possess recognizable Ehu, Ueh or Tea transporters. However, it is well known from the analysis of microbial osmostress response that bacteria often possess osmotically inducible 5-hydroxyectoine/ectoine uptake systems that are either substrate specific (e.g. EctT) or are able to import a broad spectrum of compatible solutes (e.g. ProP) (Jebbar et al., 1992; Peter et al., 1998; Vermeulen and Kunte, 2004; Kuhlmann et al., 2011).

Since our data ascribe an important regulatory function to the EnuR protein for controlling 5-hydroxyectoine/ectoine uptake and catabolic genes (Fig. 3), we searched for the presence of the *enuR* gene in the vicinity of the 5-hydroxyectoine/ectoine uptake and catabolic gene clusters. Impressively, 456 out of the 539 predicted ectoine consumers also possess *enuR* (Supporting Information Fig. S7). In an alignment of the 456 EnuR-type regulators with the *R. pomeroyi* EnuR protein as the search query, these orthologs exhibit amino sequence identities ranging between 88% (*Roseobacter* sp. SK909-2-6) to 47% (*Sinorhizobium meliloti* MVII-I). Notably, the Lys residue to which the PLP molecule is covalently attached in EnuR-type proteins (corresponding to Lys-302 of the *R. pomeroyi* EnuR protein; see Fig. 4 and Supporting Information Fig. S5) is strictly conserved (data not shown).

Phylogenetic distribution of the 5-hyxdroxyectoine/ectoine catabolic genes

Based on an amino acid sequence alignment of EutD-type proteins, we constructed a phylogenetic tree using programs provided by the iTOL web-server (Letunic and Bork, 2011). We rooted this tree with outgroup sequences of three EutD-related proteins from bacteria that do not possess 5-hydroxyectoine/ectoine catabolic gene clusters, which are represented the top-hits in the BLAST analysis outside *bona fide* EutD-type proteins. The proteins used as the out-group were a M24-type peptidase from *Mesorhizobium cicero* biovar WSM1271, a creatinase from *Pseudomonas monteilii* DSM 14164, and a Xaa-Pro aminopeptidase from *Rhizobium* sp. YR519. In stark contrast to the genes involved in ectoine and 5-hydroxyectoine synthesis that are distributed over 14 bacterial phyla (Widderich et al., 2014a; Widderich et al., 2015), genes for 5-hydroxyectoine/ectoine degradation are exclusively found within the *Proteobacteria* (Supporting Information Fig. S7).

When one views the EutD-based phylogenetic tree as a whole (Supporting Information Fig. S7), 58% of the proteins are from members of the *Alphaproteobacteria*, 14.9% from *Betaproteobacteria*, and 26.9% from *Gammaproteobacteria*. For the most part, the EutD proteins cluster with the corresponding taxonomic affiliation of the host bacteria. The very few noticeable exceptions where this is not the case can probably be explained by horizontal gene transfer, an event that in all likelihood is also responsible for the acquisition of 5-hydroxyectoine/ectoine transport and catabolic genes of the only deltaproteobacterium present in our dataset, *Desulfovibrio bastinii* DSM 16055 (Magot et al., 2004) (Supporting Information Fig. S7). It is also the only obligate anaerobe among all potential ectoine degraders that we detected. Both terrestrial and marine bacteria are represented among the 539 predicted 5-hydroxyectoine/ectoine degraders (Supporting Information Fig. S7), an observation that is expected from the taxonomic distribution of 5-hydroxyectoine/ectoine-synthesizing microorganisms that populate these habitats (Widderich et al., 2014a; Widderich et al., 2015).

The rooted tree of EutD sequences revealed that their evolution most likely originated in marine *Alphaproteobacteria*. A marine origin of 5-hydroxyectoine/ectoine degradation is highly plausible, because ectoine and 5-hydroxyectoine are mostly produced by members of the marine *Roseobacter* lineage of *Alphaproteobacteria* and by marine *Gammaproteobacteria*, which use these compounds as compatible solutes (Widderich et al., 2014a; Widderich et al., 2015). The clades of EutD sequences that appear to be closest to the common root are affiliated to strains from several orders of marine *Alphaproteobacteria*, namely the *Rhodobacterales*, *Rhizobiales*, and *Rhodospirillales* (part M1 of Supporting Information Fig. S7), as well as two families of marine *Gammaproteobacteria*, the *Vibrionaceae* and *Halomonadaceae* (sub-clusters V and H in Supporting Information Fig. S7, respectively). Because of the very different numbers of genome-sequenced strains in these phylogenetic groups, the branching order among these groups has to be treated with some caution and the ancestral sequences cannot be unequivocally identified. The alphaproteobacterial strains of group M1 are placed at the base of the tree, as defined by the outgroup sequences (group O in Supporting Information Fig. S7), but do not belong to a coherent phylogenetic group and usually represent single entities (strains, species or genera) harboring *eutD*, while their close phylogenetic neighbors do not. In contrast, the members of the two gammaproteobacterial families containing *eutD* genes appear to be phylogenetically more uniform. In particular, the EutD sequences from all sequenced strains affiliated to the genus *Halomonas* and the closely related genus *Kushneria* form a common clade (subcluster H in Supporting Information Fig. S7), as well as the sequences from many free-living strains of the *Vibrionaceae* (genera *Vibrio*, *Enterovibrio*, *Salinivibrio* and *Grimontia*; subcluster V). Most of these *Vibrionaceae* or *Halomonadeae* species

also synthesize ectoine, which could be an evolutionary link to the development of a degradative pathway. We propose from these phylogenic data of EutD that the most probable evolutionary origin of ectoine utilization has been in one of these groups of *Alpha-* or *Gammaproteobacteria*. The next clusters of *eutD* gene products branching out from the tree (group M2 in Supporting Information Fig. S7) are present in marine *Alphaproteobacteria* affiliated to the *Rhodobacterales* and *Rhodospirillales*. A further apparently contiguous large cluster of EutD sequences is found in a few species of the gammaproteobacterial family *Enterobacteriaceae* (subcluster E in Supporting Information Fig. S7) and the genus *Pseudomonas* (subcluster P in Supporting Information Fig. S7), as well as in the betaproteobacterial genus *Burkholderia* (subcluster B in Supporting Information Fig. S7). The branching order of the EutD sequences in subclusters B and P reflects very closely the evolutionary histories of the respective genera *Burkholderia* and *Pseudomonas*, based on 16S rRNA (Özen and Ussery, 2012; Santini et al., 2013). This indicates an early gene transfer of the *eut* gene cluster into the ancestors of these two genera and a long co-evolution during their radiation into the species of today.

The next branches of the pylogenetic tree again represent predominantly sequences from marine *Alphaproteobacteria* (group M3 in Supporting Information Fig. S7), indicating intensive evolutionary branching within this group. Many of these organisms belong to the "*Roseobacter* lineage" within the *Rhodobacteraceae*. However, also sequences from single strains affiliated with several other families of *Alphaproteobacteria*, as well as from the single deltaproteobacterial strain are located here, indicating further lateral gene transfer events. As a general observation, the organisms of the "*Roseobacter* lineage" comprise much less ectoine-degrading than 5-hydroxyectoine/ectoine-producing strains (Widderich et al., 2014a; Widderich et al., 2015).

Finally, the last part of the tree (Supporting Information Fig. S7) is occupied by two major sub-clusters of EutD sequences (A for *Agrobacterium* and R for *Rhizobium*) from terrestrial *Alphaproteobacteria*, which are mostly affiliated to various families of the *Rhizobiales*. Subcluster R contains a few sequence clades from strains outside the *Rhizobiales*, namely one consisting of two *Acetobacter* strains (*Rhodospirillales*) and one consisting of a number of *Rhodobacterales*, in particular several *Paracoccus* species, and is followed by large clades affiliated to the *Rhizobiales* comprising almost all genome-sequenced strains related to the genera *Mesorhizobium* (*Phyllobacteriaceae*), *Rhizobium*/*Neorhizobium*, *Ensifer*, and *Sinorhizobium* (all *Rhizobiaceae*). The branching order of the sequence from members of the *Rhizobiaceae* is again consistent with the general phylogeny of the family, suggesting long coevolution (Supporting Information Fig. S7).

The general shape of the phylogenetic tree (Supporting Information Fig. S7) suggests that the evolution of this pathway of 5-hydroxyectoine/ectoine degradation originated in marine *Alpha-* or Gamma*proteobacteria*, creating orthologs of different evolutionary stages in several orders of the contemporary marine alpha-proteobacterial population (groups M1-M3). Some of these bacteria apparently served as donors for several lateral gene transfer events, first to other marine bacteria such as the *Vibrionaceae* and *Halomonadaceae*, and later to terrestrial bacteria like the *Burkholderiaceae*, *Pseudomonadaceae*, and the terrestrial and plant-associated *Rhizobiales*. The impressive number of sequences found in these terrestrial groups is probably correlated to quite recent intensive evolutionary radiation processes. These are most likely correlated with land colonization by plants and their correlated microbial flora, especially soil-dwelling actinobacteria which are the main producers of 5-hydroxyectoine/ectoine in terrestrial habitats today (Widderich et al., 2014a; Widderich et al., 2015).

Discussion

From ecophysiological and catabolic points of view, ectoines are highly valuable nutrients since they contain two nitrogen atoms (Fig. 1C). As observed with 5-hydroxyectoine/ectoine producers that synthesize these compounds in response to osmotic stress (Widderich et al., 2014a; Widderich et al., 2015), consumers of ectoines are found in terrestrial, marine, and plant-associated ecosystems (Supporting Information Fig. S7). The co-occurrence of 5-hydroxyectoine/ectoine producers and consumers in the same habitat will ensure the supply of substrate to the 5-hydroxyectoine/ectoine degraders, and together, these two types of microorganisms will drive a biogeochemically relevant network of production and consumption of ectoines in natural environments. Ectoines are released from the microbial producer cells into the environment either upon osmotic down-shocks, or when they lyse (Booth, 2014; Welsh, 2000). Indeed, ectoines have been detected in habitats with rather different physicochemical attributes: soils and acid mine drainage solutions (Mosier et al., 2013; Warren, 2014).

Before 5-hydroxyectoine or ectoine can be exploited as nutrients, they must be efficiently retrieved from environmental resources. Our bioinformatics assessment of the genome context of 5-hydroxyectoine/ectoine consumers revealed that 418 out of the inspected 539 strains possess genes for either an EhuABCD-type ABC transporter (Hanekop et al., 2007), or for the UehABC-type TRAP transporter (Lecher et al., 2009) within the catabolic gene cluster (Fig. 5 and Supporting Information Fig. S7). In addition, a number of microorganisms possess genes for TRAP transporters related to the 5-hydroxyectoine/ectoine-specific TeaABC transporter from *H. elongata*

(Grammann et al., 2002) somewhere else in their genomes. Although the EhuABCD ABC transporter and the UehABC/TeaABC TRAP-type uptake systems belong to different transporter families (Davidson et al., 2008; Mulligan et al., 2011), each of them relies on a periplasmic ligand-binding protein (EhuB, UehA, TeaA) that binds ectoines avidly with K_d-values in the low μM range (Hanekop et al., 2007; Lecher et al., 2009; Kuhlmann et al., 2008). Hence, the EhuABCD, UehABC and TeaABC transporters are well suited to effectively scavenge ectoines from scarce environmental sources.

Our *in silico* assessment of the taxonomic distribution of predicted 5-hydroxyectoine/ectoine consumers revealed their exclusive affiliation with the *Proteobacteria* (Supporting Information Fig. S7). This is in stark contrast to the taxonomy of 5-hydroxyectoine/ectoine producers that are associated with 14 bacterial and archaeal phyla (Widderich et al., 2014a; Widderich et al., 2015). An evolutionarily highly conserved *ectABC(D)* biosynthetic operon is found in most 5-hydroxyectoine/ectoine producers (Widderich et al., 2014a; Widderich et al., 2015). It is thus striking how variable the arrangements of the 5-hydroxyectoine/ectoine catabolic genes are (Fig. 5). Some of these gene clusters contain genes that are not present in those found in *S. meliloti* (Jebbar et al., 2005) or *R. pomeroyi* (Lecher et al., 2009) but that are nevertheless repeatedly observed (Fig. 5). Examples are the replacement of *eutE* (Fig. 1C) with *argE* like genes (annotated as a *N*-acetylornithine deacetylase), and the presence of genes encoding for a NADPH:quinone reductase and a γ-glutamyltransferase, respectively (Fig. 5). Furthermore, a considerable variation exists with respect to the presence of the *eutA* gene, whose encoded product is envisioned to alter the stereochemistry of 5-(*S*)-hydroxyectoine to form 5-(*R*)-hydroxyectoine (Fig. 1C and Supporting Information Fig. S4). Taken together, these observations suggest that the 5-hydroxyectoine/ectoine catabolic gene clusters are still undergoing evolutionary changes and suggest that variations of the 5-hydroxyectoine/ectoine catabolic route that we propose here for *R. pomeroyi* (Fig. 1C) are likely to exist in other bacteria.

Since ectoines are unlikely to be present all the time in a given habitat (Mosier et al., 2013; Warren, 2014), it makes physiologically sense for nutrient-limited microorganisms to exert a tight transcriptional control over their 5-hydroxyectoine/ectoine uptake and catabolic genes. In agreement with initial data reported for *S. meliloti* (Jebbar et al., 2005), expression of the corresponding genes from *R. pomeroyi* are substrate inducible (Supporting Information Fig. S2) and are transcribed as a 13.5 kb gene cluster (Fig. 1A and B). Two differently regulated promoters mediate the expression of this operon. The promoter present in front of *enuR* is weak and does not respond to ectoine availability (Fig. 3A). In contrast, the promoter present in front of the *uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster is ectoine inducible (about 36-fold) in an EnuR-dependent fashion, a regulatory protein serving as a repressor (Fig. 3A). Substrate-induction of the *uehABC-usp-eutABCDE-asnC-ssd-atf* operon triggers enhanced UehABC-mediated ectoine import (Lecher et al., 2009) and will thereby further contribute to the transcriptional induction of the 5-hydroxyectoine/ectoine uptake and catabolic genes in *R. pomeroyi* until the environmental 5-hydroxyectoine and ectoine resources are depleted.

Genes for EnuR-related proteins are found in 456 strains (out of 539) in the vicinity of 5-hydroxyectoine/ectoine uptake and catabolic gene clusters (Supporting Information Fig. S7), implying a major role for this regulatory protein in controlling 5-hydroxyectoine/ectoine degradation. EnuR is a member of the GntR superfamily of transcriptional regulators (Rigali et al., 2002) and belongs to the MocR/GabR subfamily (Bramucci et al., 2011; Milano et al., 2015; Suvorova and Rodionov, 2016). Our modeling studies indicate that EnuR adheres to the design principles common to MocR/GabR-type regulators with their N-terminal DNA-binding domain connected *via* a long flexible linker region to a large C-terminal domain resembling the fold of aminotransferase I enzymes (Supporting Information Fig. S5) (Edayathumangalam et al., 2013; Okuda et al., 2015a; Okuda et al., 2015b). The aminotransferase I domain of MocR/GabR-type regulators averages 300 amino acids in size and the so far experimentally studied representatives carry a covalently attached PLP molecule (Bramucci et al., 2011; Milano et al., 2015; Suvorova and Rodionov, 2016). However, despite the resemblance of this domain to *bona fide* aminotransferases [e.g., LysN from *Thermus thermophilus*; (Tomita et al., 2009)], MocR/GabR-type regulators do not carry out a full aminotransferase enzyme reaction (Edayathumangalam et al., 2013; Milano et al., 2015; Okuda et al., 2015a; Okuda et al., 2015b; Suvorova and Rodionov, 2016). Nevertheless, the attached PLP molecule is critical for the role of these proteins as transcriptional regulators, probably by invoking a conformational change of the regulatory protein upon the chemical reaction of PLP with a system-specific inducer molecule (Belitsky and Sonenshein, 2002; Belitsky, 2004; Bramucci et al., 2011; Milano et al., 2015; Okuda et al., 2015a; Okuda et al., 2015b; Suvorova and Rodionov, 2016).

Our biochemical analysis of the purified EnuR protein shows that it indeed contains a covalently attached PLP molecule (Fig. 4) and modeling and site-directed mutagenesis studies identified Lys-302 as the residue to which PLP is bound (Supporting Information Fig. S5). This residue is strictly conserved in an amino acid sequence alignment of all 456 EnuR-type proteins identified in our *in silico* analysis (data not shown). As revealed by in depth studies with the activator and auto-repressor GabR from *Bacillus*

subtilis, the addition of the inducer GABA leads to the cleavage of the covalent bond formed between Lys-312 of GabR and the PLP co-factor; simultaneously a new covalent bond is formed between the primary amino group of the inducer GABA and PLP (Edayathumangalam et al., 2013; Okuda et al., 2015a; Okuda et al., 2015b). For the functioning of this type of chemistry, a primary amino group of the inducer molecule is required. Although exogenously provided ectoine is a strong inducer of the transcription of the 5-hydroxyectoine/ectoine uptake and catabolic genes (Supporting Information Fig. S2 and Fig. 3A), it lacks a primary amino group that could chemically interact with the PLP cofactor to form a covalent bond (Fig. 1C). Based on these considerations, we suggest that ectoine is not the true inducer of the 5-hydroxyectoine/ectoine uptake and catabolic genes. Instead, we propose that an internal inducer is generated through catabolism from the imported 5-hydroxyectoine and ectoine molecules, which then affects the DNA-binding activity of EnuR. Indeed, the catabolism of ectoine generates several compounds that could potentially chemically interact with the EnuR-bound PLP molecule (Fig. 1C).

Many of the 5-hydroxyectoine/ectoine uptake and catabolic gene clusters contain an *asnC* gene (Fig. 5), and notably, a *R. pomeroyi asnC* mutant can no longer exploit ectoines as nutrients (Fig. 2F). AsnC-type regulators belong to the "feast and famine" group of transcriptional regulators (Deng et al., 2011). They form oligomers (often octamers) wrapping the target DNA around them and their DNA-binding properties are dictated by small molecules, mostly amino acids (Kumarevel et al., 2008; Deng et al., 2011; Dey et al., 2016). An *asnC*-type gene (*deoX*) is also present in the ectoine catabolic gene cluster of *H. elongata* (Fig. 5). Preliminary DNA binding studies with the corresponding AsnC (DoeX)-type protein shows that it can bind to a DNA fragment positioned at the 5' end of the *eutD* (*doeA*) gene (Schwibbert et al., 2011). Our finding that the inactivation of the *asnC* gene in *R. pomeroyi* DSS-3 abolishes the use 5-hydroxyectoine/ectoine as nutrients (Fig. 2F) suggests an important function for this type of "feast and famine" regulator for the catabolism of ectoines.

Taken our findings together, the genetic circuit(s) for the catabolism of ectoines seem to be complex and finely tuned. It will therefore be of considerable interest to unravel the interplay between the EnuR and AsnC regulatory proteins and to identify their corresponding effector molecule(s). The data presented here provide a solid conceptual framework for the understanding of the use of ectoines as nutrients by a substantial, but taxonomically restricted, group of terrestrial and marine microorganisms. They also suggest avenues for the further dissection of the genetic regulatory circuits controlling the 5-hydroxyectoine/ectoine catabolic system.

Experimental procedures

Chemicals

Ectoine and 5-hydroxyectoine were kindly provided by the bitop AG (Witten, Germany). Anhydrotetracycline hydrochloride (AHT), desthiobiotin, and Strep-Tactin Superflow chromatography material were purchased from IBA GmbH (Göttingen, Germany). The substrate for β-galactosidase, o-nitrophenyl-β-*D*-galactopyranosid (ONPG), was obtained from Serva (Heidelberg, Germany). The antibiotics kanamycin, gentamycin, spectinomycin, and rifampicin were purchased from Serva; ampicillin was obtained from Carl Roth GmbH (Karlsruhe, Germany).

Bacterial strains and plasmids

The *Escherichia coli* strain DH5α was used for routine cloning purposes and the *E. coli* B strain BL21(DE3) (Stratagene, La Jolla, CA) was used for the overexpression of the *R. pomeroyi enuR* gene. The *R. pomeroyi* strain DSS-3 (Moran et al., 2004) was obtained from the German Collection of Microorganisms (DSMZ; Braunschweig, Germany) and a rifampicin-resistant (Rif^R) derivative (strain J470) (Todd et al., 2012a) was kindly provided by J. Todd and A. Johnston (University of East Anglia, United Kingdom). These colleagues also generously provided an *E. coli* DH5α strain carrying the helper plasmid pRK2013 [Kan^R] (Figurski and Helinski, 1979) used for tri-parental conjugation experiments between *E. coli* (pRK2013), the *R. pomeroyi* strain J470, and *E. coli* strains carrying the *lacZ* reporter fusion plasmid pBIO1878 [$Spec^R$] (Todd et al., 2012a) or its derivatives pBAS19 and pBAS21, respectively. The suicide vector pK18mobsacB [Kan^R] (Kvitko and Collmer, 2011) used for the construction of chromosomal deletions in *R. pomeroyi* (Lidbury et al., 2014) was kindly provided by I. Lidbury and Y. Chen (University of Warwick, United Kingdom). Plasmid p34S_Gm carries a gentamycin resistance cassette (Dennis and Zylstra, 1998), which was used as a selection marker for chromosomal deletion/insertion mutations in *R. pomeroyi*; it was provided by I. Lidbury and Y. Chen as well. pK18mobsacB derivatives were introduced into *R. pomeroyi* strain J470 by tri-parental conjugation experiments as described above.

Media and growth conditions

Strains of *E. coli* were routinely propagated and maintained aerobically on Luria Bertani (LB) agar plates (Miller, 1992) at 37°C. Ampicillin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}), spectinomycin (50 μg mL^{-1}) and gentamycin (20 μg mL^{-1}) were added to solid and liquid growth media for *E. coli* strain to select for recombinant plasmids. For the overproduction of the EnuR protein, cells of the *E. coli* B strain BL21(DE3), were grown in Minimal Medium A (MMA) (Miller, 1972), supplemented with 0.4% (w/v) casamino acids, 0.4% (w/v) glucose, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mg L^{-1} thiamine and 100 μg ampicillin to select for the *enuR* expression plasmid pBAS3. *R. pomeroyi* DSS-3 and its Rif^R derivative strain J470 were routinely propagated on ½ YTSS agar plates (Todd et al., 2012b). For growth experiments in liquid media, *R. pomeroyi* was cultivated in defined basal media (Baumann et al., 1971)

that was prepared with some modifications. The medium used had the following composition: 50 mM MOPS (pH 7.5), 200 mM NaCl, 10 mM KCl, 330 mM K_2HPO_4, 10 mM $CaCl_2$, 50 mM $MgSO_4$, 0,1 mM $FeSO_4$, and it contained 200 mM NH_4Cl as the nitrogen source. To this basal medium, we added a 200-fold diluted solution of vitamin mixture A and vitamin mixture B, respectively. The 200 x concentrate of vitamin mixture A contained 39 $\mu g\ L^{-1}$ biotin, 78 $\mu g\ L^{-1}$ nicotinic acid, 78 $\mu g\ L^{-1}$ lipoic acid and 78 $\mu g\ L^{-1}$ folic acid. The 200 x concentrate of vitamin mixture B contained 78 $\mu g\ L^{-1}$ pantothenic acid, 78 $\mu g\ L^{-1}$ pyridoxine, 78 $\mu g\ L^{-1}$ thiamine, 78 $\mu g\ L^{-1}$ 4-aminobenzoic acid and 1.6 $\mu g\ L^{-1}$ cobalamin. We added to the growth medium also 0.1 mM methionine, 0.1 mM serine and 0.1 mM glutamate since we found that these additives considerably enhanced the growth of *R. pomeroyi* DSS-3 and its derivatives. Glucose (28 mM) was routinely used as the carbon source for *R. pomeroyi* DSS-3 in this chemically defined medium. When either ectoine or 5-hydroxyectoine was used as combined carbon and nitrogen sources, NH_4Cl was left out from the medium and these compounds were provided at a final concentration of 28 mM. When required, the antibiotics kanamycin, gentamycin, spectinomycin and rifampicin were used at the following concentrations: 80 $\mu g\ mL^{-1}$, 20 $\mu g\ mL^{-1}$, 150 $\mu g\ mL^{-1}$, and 20 $\mu g\ mL^{-1}$, respectively.

DNA isolation and recombinant DNA techniques

Chromosomal DNA of *R. pomeroyi* DSS-3 was isolated according to Marmur (Marmur, 1961). Plasmid DNA was isolated from *E. coli* strains using the High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany). Restriction endonucleases and DNA ligase were purchased from ThermoScientific (St. Leon-Rot, Germany) and were used under conditions recommended by the manufacturer. Chemically competent cells of *E. coli* were prepared and transformed as described previously (Sambrook et al., 1989).

Construction of plasmids and bacterial strains

The *lacZ* transcriptional reporter gene fusion plasmids pBAS19 and pBAS21 were constructed as follows. For pBAS19 (*enuR-lacZ*), a 1.1 kb DNA fragment encompassing 1097 bp upstream of *enuR* and the first 22 amino acids of the *enuR* gene (Fig. 4A) was amplified from genomic DNA of *R. pomeroyi* DSS-3 using Phusion DNA polymerase (Life Technologies, Darmstadt, Germany) and the custom-synthesized DNA primers LacZenuR_up_for and LacZenuR_PstI_rev (Supporting Information Table S2). The chromosomal 0.5 kb DNA fragment present in pBAS21 (*uehA-lacZ*) includes 308 bp of the 5' truncated *enuR* gene, the 97 bp *enuR-uehA* intergenic region and 28 bp of the *uehA* gene (Fig. 4A). This DNA segment was amplified with the aid of the LacZuehA_EcoRI_ for and LacZuehA_PstI_revprimers (Supporting Information Table S2). The amplified DNA fragments were purified using the QIA Quick PCR Purification Kit (Qiagen, Hilden, Germany), digested with EcoRI and PstI and ligated into the broad-host-range *lacZ*-fusion vector pBIO1878 (Todd et al., 2012a) that had been linearized with the same restriction endonucleases.

To construct the Δ(*enuR-uehABC-usp-eutABCDE-asnC-ssd-atf::Gm*)1 (strain ASR6), Δ(*enuR::Gm*)1 (strain ASR7), Δ(*eutD::Gm*)1 (strain ASR8), Δ(*asnC-ssd-atf::Gm*)1 (strain ASR10), Δ(*eutABC::Gm*)1 (strain ASR11), Δ*(eutABCDE-asnC-ssd-atf::Gm*)1 (strain ASR12), and Δ(*ssd-atf::Gm*)1 (strain ASR14) mutants of the *R. pomeroyi* strain J470, 600 bp fragments located upstream and downstream of the respective genomic areas (Fig. 1A) were amplified by PCR using custom synthesized primers (Supporting Information Table S2). A gentamycin resistance cassette was amplified with PCR from p34S_Gm (Dennis and Zylstra, 1998) (Supporting Information Table S1), and all three DNA fragments were then cloned into the suicide vector pK18mobsacB (linearized with EcoRI/XbaI) using the Gibson assembly procedure (Gibson et al., 2009). This yielded plasmids pBAS24 [Δ(*enuR-uehABC-usp-eutABCDE-asnC-ssd-atf::Gm*)1], pBAS34 Δ(*enuR::Gm*)1], pBAS35 Δ(*eutD::Gm*)1], pBAS42 [Δ(*asnC-ssd-atf::Gm*)1], pBAS44 [Δ*(eutABCDE-asnC-ssd-atf::Gm*)1], pBAS45 [Δ(*eutABC::Gm*)1], and pBAS49 [Δ(*ssd-atf::Gm*)1], respectively. In the Δ(*enuR-uehABC-usp-eutABCDE-asnC-ssd-atf::Gm*)1 mutation, the entire 5-hydroxyectoine/ectoine uptake and catabolic gene cluster (14,220 bp) is removed. The Δ(*enuR::Gm*)1 deletion removes 975 bp (corresponding to amino acids of EnuR from 1 to 325). The Δ(*eutABC::Gm*)1 mutation removes 2,775 bp starting at the ATG start codon of *eutA* and finishing with the TGA stop codon of *eutC*. In the Δ(*eutD::Gm*)1 deletion mutant a DNA sequence spanning the whole *eutD* gene (1,188 bp) is removed from the operon. The Δ(*asnC-ssd-atf::Gm*)1 mutation removes 3,611 bp, which begins at the ATG start codon of *asnC* and ends at TGA stop codon of *atf*. The Δ(*ssd-atf::Gm*)1 deletion includes the DNA sequence from ATG of *ssd* to TGA of *atf* (3,004 bp). In the Δ(*eutABCDE-asnC-ssd-atf::Gm*)1 mutation, 8,603 bp are removed from the operon, starting at the ATG of *eutA* and ending at TGA of *atf*.

To transfer plasmids from the Rif^S *E. coli* strains into the Rif^R *R. pomeroyi* strain J470, a tri-parental mating experiment was set up. The *E. coli* strain DH5α carrying the helper plasmid pRK2013, strain DH5α carrying the plasmid destined for transfer into *R. pomeroyi* and *R. pomeroyi* J470, were grown to early stationary phase in media [the *E. coli* strains were propagated in LB; *R. pomeroyi* was grown in ½ YTSS] lacking antibiotics. Cells were harvested by centrifugation (4000*g*) and cell pellets of 2 mL *R. pomeroyi* culture and 1 mL of each *E. coli* culture were re-suspended and mixed in 100 μL ½ YTSS medium. This cell mixture was then spotted onto 595 S&S filter papers (Schleicher&Schuell, Germany) and placed on ½ YTSS agar plates to allow conjugation; the agar plates were incubated at 30˚C. After incubation for 24 h, cells were retrieved from the filters, re-suspended in 1 mL ½ YTSS medium and 10- and 100-μL aliquots were plated on minimal agar plates prepared from the above described chemically defined medium for *R. pomeroyi* containing appropriate antibiotics; the plates were incubated for 2–3 days at 30˚C. Colonies were then tested for their antibiotic resistance/sensitivity pattern by patching them onto ½ YTSS agar plates. This procedure was used both for plasmids that can replicate in *R. pomeroyi* (derivatives of the *lacZ* fusion vector pBIO1878) or can integrate into its genome (derivatives of the suicide vector pK18mobsacB). Strains of *R. pomeroyi* J470 carrying replicative plasmids derived from pBIO1878 are Rif^R and $Spec^R$.

R. pomeroyi J470 strains carrying the desired chromosomal gene disruption mutation after the tri-prarental mating, are Rif^R Kan^S (loss of the pK18mobsacB backbone) and Gm^R (indicating the presence of the chromosomal deletion/insertion mutation).

To construct the expression plasmid for the *R. pomeroyi* EnuR protein attached to a carboxy-terminal *Strep*-tag-II affinity peptide, the *enuR* gene was codon optimized for its expression in *E. coli* (Life Technologies, Darmstadt, Germany) with added LguI restriction sites on either ends. The synthetic gene (GenBank accession number KU891821) was liberated from the plasmid provided by the supplier by cleaving the DNA with SfiI. The obtained synthetic DNA fragment was cloned into the donor vector pENTRY-IBA20 (IBA GmbH; Göttingen, Germany) via LguI restriction and subsequent ligation; the resulting plasmid was pBAS2. For the subsequent step, the IBA Stargate cloning system was used. DNA of plasmid pBAS2 was cut with Esp3I and concurrent ligation of the liberated DNA fragment into the expression vector pASG-IBA3 was carried out. In the resulting plasmid pBAS3, the 3' end of the *enuR* coding region is fused to a short DNA fragment encoding the nine amino acids comprising *Strep*-tag-II affinity peptide. Expression of recombinant *enuR* gene in pBAS3 is mediated by the TetR-controlled *tet* promoter carried by the backbone of plasmid pASG-IBA3. Adding the inducer for the TetR repressor, AHT, to the growth medium, triggers induction of *enuR* transcription in *E. coli* cells carrying pBAS3. The correct nucleotide sequence of the *enuR* gene and its flanking regions was ascertained by DNA sequencing, which was carried out by Eurofins MWG Operon (Ebersberg, Germany).

To confirm the covalent binding of the PLP to the wild-type EnuR protein, site-directed mutagenesis was performed using the *enuR* gene of *R. pomeroyi* present in pBAS3. The QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and custom-synthesized mutagenic primers (Microsynth, Lindau, Germany) were utilized to alter Lys^{302} into a His residue [AAA → CAT]. The presence of the desired mutation and the absence of undesired alterations in plasmid pBAS17 were confirmed by DNA sequencing (Eurofins MWG GmbH, Ebersberg, Germany).

β-galactosidase enzyme assays

The *lacZ* reporter gene plasmids pBAS19 and pBAS21 (Fig. 4A) were introduced into the *R. pomeroyi* strain J470 via conjugation. 25-mL cultures of the corresponding plasmid-containing strains were grown at 30° C for 24 h (they reached by that time an OD_{578} of about 1.0) in an aerial shaker (set to 200 rpm) with glucose (28 mM) as the carbon source and NH_4Cl was present as the nitrogen source (for the control plasmid pBIO1878 and the *lacZ* reporter gene plasmids pBAS19 and pBAS21). Cells harboring the *lacZ* reporter gene plasmids pBAS19 (*enuR-lacZ*) and pBAS21 (*uehA-lacZ*) were grown with ectoine (28 mM) as the sole carbon and nitrogen source in the modified minimal medium described above. The medium contained spectinomycin (150 μg mL^{-1}) to select for the presence of the reporter plasmids. Cells were harvested by centrifugation (13 000 rpm for 10 min) and assayed for β-galactosidase activity as described (Miller, 1972). β-galactosidase enzyme activity is expressed as Miller Units (MU). Cells of *R. pomeroyi* strain J470 carrying the empty pBIO1878 *lacZ* reporter vector was used as a control and had a background β-galactosidase activity of 1 to 2 Miller units.

Overproduction, purification and quaternary assembly of the EnuR regulatory protein

A pre-culture of the *E. coli* B strain BL21 (DE3) carrying the *enuR* overexpression plasmid pBAS3 was grown overnight in LB medium containing ampicillin (100 μg mL^{-1}) and used to inoculate 1 L of MMA to an OD_{578} of about 0.05. The cells were grown at 37°C in an aerial shaker (set to 220 rpm) until the culture reached mid-log phase (OD_{578} of about 0.6). Enhanced expression of the synthetic *enuR* gene carried by plasmid pBAS3 was triggered by adding the inducer of the TetR repressor, AHT, to a final concentration of 0.2 mg mL^{-1} to the growth medium. At this point, the growth temperature was lowered to 35°C and the cells were grown for an additional 2 h. Bacteria were collected by centrifugation (20 min at 4°C, 5000 rpm) and the cell pellets were stored at −20°C for future use. For the purification of the EnuR-*Strep* tag-II protein, the overproducing cells were re-suspended in a buffer containing 10 mM Tris (pH 7.5) and 150 mM NaCl (buffer A), and lysed by passing them three times through a French Press (Aminco, Silver Spring, USA) at 1000 psi. Cell debris was removed by centrifugation for 1 h at 35,000 rpm and 4°C. The cleared cell lysate was then passed through a Strep-Tactin Superflow column that had been equilibrated with buffer A. The column was washed with five bed volumes of buffer A and the proteins bound by the Strep-Tactin Superflow material were eluted with buffer A containing desthiobiotin (0.46 mg mL^{-1}). The purity of the purified EnuR-*Strep* tag-II protein was assessed by SDS-polyacrylamide gel electrophoresis using 15% polyacrylamide gels. The quaternary assembly of the isolated EnuR-*Strep* tag-II protein in solution was studied by SEC. For these experiments a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Europe GmbH, Freiburg, Germany) connected to an ÄKTA pure 25 L system (GE Healthcare Europe GmbH) was used. For column equilibration and the run, a 10 mM Tris buffer (pH 7.5) containing 150 mM NaCl was used; 6 mg of the EnuR-*Strep* tag-II protein was loaded onto the size-exclusion column. To assess and standardize the performance of the size-exclusion column, a protein solution consisting of [3 mg mL^{-1}] of carbonic anhydrase (from bovine erythrocytes) (29 kDa), albumin (from bovine serum) (66 kDa), and alcohol dehydrogenase (from *Saccharomyces cerevisiae*) (150 kDa) was used (Gel Filtration Markers Kit; Sigma-Aldrich, St. Louis, MO). The column run of the EnuR-*Strep* tag-II protein and the marker proteins was evaluated with the Unicorn 6.3 software package (GE Healthcare Europe GmbH) to calculate the apparent quaternary assembly of the EnuR protein in solution. The EnuR (Lys-302/His) variant protein was overproduced in BL21 (DE3) cells carrying plasmid pBAS17 and purified and analyzed by SEC as described for the wild-type EnuR protein. Concentrations of the EnuR protein were estimated in a nano-drop spectrophotometer (model ND-1000; Thermo-Scientific, Braunschweig, Germany) using a molecular mass of 51.977 kDa (including the *Strep* tag II peptide) and a molar extinction co-efficient of 64 775 M^{-1} cm^{-1}.

Isolation of total RNA and transcription analysis of the 5-hydroxyectoine/ectoine uptake and catabolic gene cluster

Total RNA was isolated from early log phase cells of *R. pomeroyi* DSS-3 grown at 30° C in basal minimal medium containing 28 mM glucose, ectoine, or 5-hydroxyectoine, respectively, as the carbon source. NH_4Cl was present as the nitrogen source. The RNA was isolated by a modified acidic phenol method (Völker et al., 1994). In order to analyze whether the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* genes (Fig. 1A) are transcribed as a unit, nine intergenic regions of the putative operon were amplified from isolated RNA using the Qiagen one Step RT-PCR Kit and gene-specific DNA primers (Supporting Information Table S2). To recognize PCR products that resulted from DNA contamination in the RNA sample, a control was performed in which total RNA was added after the reverse transcription step. The size of the generated RT-PCR products was analyzed by agarose (1%) gel electrophoresis.

Transcriptional analyses by the northern dot blot technique

A potential increase in the level of transcription of the *R. pomeroyi* DSS-3 *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf*gene cluster in response to the presence of ectoine or 5-hydroxyectoine in the growth medium was assessed by dot blot hybridization analyses. Different concentrations (3 μg-180 ng) of total RNA prepared from *R. pomeroyi* DSS-3 cells grown in basal medium containing either 28 mM glucose, ectoine or 5-hydroxyectoine as the sole carbon source was spotted onto a nylon membrane (NytranN; Schleicher& Schuell) using a dot blot apparatus (Bio-Rad Laboratories GmbH). For the detection of specific *R. pomeroyi* DSS-3 genes, the total RNA was hybridized to digoxigenin-labeled RNA probes at 68°C and the membranes were processed and developed according to the manual of the Dig Northern Starter Kit (Roche Diagnostic GmbH). Digoxigenin-labeled antisense RNA probe were prepared by *in vitro* transcription of PCR templates containing the T7-RNA polymerase promoter sequence using the Dig Northern Starter Kit. The intensity of the hybridization signal was quantified using a Storm860 fluorescence imager and the ImageQuant software package (Amersham Pharmacia Biotech).

Database searches for EutD*-related proteins*

Proteins homologous to the EutD protein (accession number: AAV94440.1) from *R. pomeroyi* DSS-3 (Moran et al., 2004) were searched via the Web server of the genome portal of the Department of Energy Joint Genome Institute (http://genome.jgi.doe.gov/) (JGI) (Nordberg et al., 2013) using the BLAST algorithm (Altschul et al., 1990) (search date: 06.01.2016). The genome context of finished and unfinished microbial genomes in the vicinity of *eutD*-type genes was assessed using the gene neighborhood tool (http://img.jgi.doegov/cgi-bin/pub/main.cgi) provided by the JGI Web-server. Only *eutD* gene homologs that showed two or more of the other *eutABCE_ssd_atf* catabolic genes in the direct vicinity were selected as potential ectoine degraders and used for further analyses. Amino acid sequence alignments of orthologs of the *R. pomeroyi* DSS-3 EutD protein were performed with ClustalΩ (Larkin et al., 2007), and the ClustalΩ output was used for construction of a phylogenetic tree using the online tool Interactive Tree of Life (http://itol.embl.de/) (Letunic and Bork, 2011). Subsequently, the gene organization of the clusters found, were individually inspected using the online tool available from the DOE Joint Genome Institute website (Nordberg et al., 2013). Searches were carried out for the presence of genes coding for 5-hydroxyectoine/ectoine transporters (UehABC, EhuABCD, TeaABC), the *enuR* gene, the presence of *eutABC* genes or other additional genes (γ-glutamyltransferase or a NADPH:Quinone reductase) in the direct vicinity of the gene catabolic gene cluster. An additional BLAST search (Altschul et al., 1990) via the Web-server of the genome portal of the Department of Energy Joint Genome Institute (http://genome.jgi.doe.gov/) (JGI) (Nordberg et al., 2013) was performed, searching for genomes containing an 5-hydroxyectoine/ectoine degradation gene cluster for the presence of ectoine and 5-hydroxyectoine synthetic genes (Widderich et al., 2014a; Widderich et al., 2015). Homologues to the EctC (accession number: AAY29688) and EctD (accession number: AAY29689) proteins from *Virgibacillus salexigens* were used as the query sequence.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Baumann, P., Baumann, L., and Mandel, M. (1971) Taxonomy of marine bacteria: the genus *Beneckea*. *J Bacteriol* **107:** 268–294.

Belitsky, B.R. (2004) *Bacillus subtilis* GabR, a protein with DNA-binding and aminotransferase domains, is a PLP-dependent transcriptional regulator. *J Mol Biol* **340:** 655–664.

Belitsky, B.R., and Sonenshein, A.L. (2002) GabR, a member of a novel protein family, regulates the utilization of gamma-aminobutyrate in *Bacillus subtilis*. *Mol Microbiol* **45:** 569–583.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T, *et al*. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* **42:** W252–W258.

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

Boyen, A., Charlier, D., Charlier, J., Sakanyan, V., Mett, I., and Glansdorff, N. (1992) Acetylornithine deacetylase, succinyldiaminopimelate desuccinylase and carboxypeptidase G2 are evolutionarily related. *Gene* **116:** 1–6.

Bramucci, E., Milano, T., and Pascarella, S. (2011) Genomic distribution and heterogeneity of MocR-like transcriptional factors containing a domain belonging to the superfamily of the pyridoxal-5'-phosphate dependent enzymes of fold type I. *Biochem Biophys Res Commun* **415:** 88–93.

Bremer, E., and Krämer, R. (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes. In *Bacterial Stress Responses*. Storz, G., and Hengge-Aronis, R. (eds). Washington DC, USA: ASM Press, pp. 79–97.

Brinkhoff, T., Giebel, H.A., and Simon, M. (2008) Diversity, ecology, and genomics of the *Roseobacter* clade: a short overview. *Arch Microbiol* **189:** 531–539.

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

Christen, P., and Mehta, P.K. (2001) From cofactor to enzymes. The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. *Chem Rec* **1:** 436–447.

Davidson, A.L., Dassa, E., Orelle, C., and Chen, J. (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* **72:** 317–364.

Deng, W., Wang, H., and Xie, J. (2011) Regulatory and pathogenesis roles of *Mycobacterium* Lrp/AsnC family transcriptional factors. *J Cell Biochem* **112:** 2655–2662.

Dennis, J.J., and Zylstra, G.J. (1998) Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl Environ Microbiol* **64:** 2710–2715.

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

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

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* **76:** 1648–1652.

Galinski, E.A., and Herzog, R.M. (1990) The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). *Arch Microbiol* **153:** 607–613.

Gallagher, D.T., Gilliland, G.L., Xiao, G., Zondlo, J., Fisher, K.E., Chinchilla, D., and Eisenstein, E. (1998) Structure and control of pyridoxal phosphate dependent allosteric threonine deaminase. *Structure.* **6:** 465–475.

Gallagher, D.T., Monbouquette, H.G., Schröder, I., Robinson, H., Holden, M.J., and Smith, N.N. (2004) Structure of alanine dehydrogenase from *Archaeoglobus*: active site analysis and relation to bacterial cyclodeaminases and mammalian mu crystallin. *J Mol Biol* **342:** 119–130.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A. III, and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* **6:** 343–345.

Goodman, J.L., Wang, S., Alam, S., Ruzicka, F.J., Frey, P.A., and Wedekind, J.E. (2004) Ornithine cyclodeaminase: structure, mechanism of action, and implications for the mu-crystallin family. *Biochemistry* **43:** 13883–13891.

Grammann, K., Volke, A., and Kunte, H.J. (2002) New type of osmoregulated solute transporter identified in halophilic members of the *Bacteria* domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM 2581(T). *J Bacteriol* **184:** 3078–3085.

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

Hoffmann, T., Boiangiu, C., Moses, S., and Bremer, E. (2008) Responses of *Bacillus subtilis* to hypotonic challenges: physiological contributions of mechanosensitive channels to cellular survival. *Appl Environ Microbiol* **74:** 2454–2460.

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

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

Jebbar, M., Talibart, R., Gloux, K., Bernard, T., and Blanco, C. (1992) Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. *J Bacteriol* **174:** 5027–5035.

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

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

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

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

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

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

Kvitko, B.H., and Collmer, A. (2011) Construction of *Pseudomonas syringae* pv. Tomato DC3000 mutant and polymutant strains. *Methods Mol Biol* **712:** 109–128.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., *et al*. (2007) Clustal W and clustal X version 2.0. *Bioinformatics* **23:** 2947–2948.

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

Letunic, I., and Bork, P. (2011) Interactive tree of life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* **39:** W475–W478.

Levina, N., Totemeyer, S., Stokes, N.R., Louis, P., Jones, M.A., and Booth, I.R. (1999) Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. *EMBO J* **18:** 1730–1737.

Lidbury, I., Murrell, J.C., and Chen, Y. (2014) Trimethylamine N-oxide metabolism by abundant marine heterotrophic bacteria. *Proc Natl Acad Sci U S A* **111:** 2710–2715.

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

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

Luo, H., and Moran, M.A. (2014) Evolutionary ecology of the marine *Roseobacter* clade. *Microbiol Mol Biol Rev* **78:** 573–587.

Magot, M., Basso, O., Tardy-Jacquenod, C., and Caumette, P. (2004) *Desulfovibrio bastinii* sp. nov. And *desulfovibrio gracilis* sp. nov., moderately halophilic, sulfate-reducing bacteria isolated from deep subsurface oilfield water. *Int J Syst Evol Microbiol* **54:** 1693–1697.

Manzanera, M., Garcia de Castro, A., Tondervik, A., Rayner-Brandes, M., Strom, A.R., and Tunnacliffe, A. (2002) Hydroxyectoine is superior to trehalose for anhydrobiotic engineering of *Pseudomonas putida* KT2440. *Appl Environ Microbiol* **68:** 4328–4333.

Marmur, J. (1961) Procedure for isolation of dioxyribonucleic acid from micrroganism. *J Mol Biol* **3:** 208–218.

Milano, T., Contestabile, R., Lo Presti, A., Ciccozzi, M., and Pascarella, S. (2015) The aspartate aminotransferase-like domain of *Firmicures* MocR transcriptional regulators. *Comput Biol Chem* **58:** 55–61.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Miller, J.H. (1992) *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

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

Mosier, A.C., Justice, N.B., Bowen, B.P., Baran, R., Thomas, B.C., Northen, T.R., and Banfield, J.F. (2013) Metabolites associated with adaptation of microorganisms to an acidophilic, metal-rich environment identified by stable-isotope-enabled metabolomics. *Mbio* **4:** e00484–e00412.

Mulligan, C., Fischer, M., and Thomas, G.H. (2011) Tripartite ATP-independent periplasmic (TRAP) transporters in *Bacteria* and *Archaea*. *FEMS Microbiol Rev* **35:** 68–86.

Nordberg, H., Cantor, M., Dusheyko, S., Hua, S., Poliakov, A., Shabalov, I., *et al*. (2013) The genome portal of the department of energy joint genome institute: 2014 updates. *Nucleic Acids Res* **42:** D26–D31.

Okuda, K., Ito, T., Goto, M., Takenaka, T., Hemmi, H., and Yoshimura, T. (2015a) Domain characterization of *Bacillus* subtilis GabR, a pyridoxal 5′-phosphate-dependent transcriptional regulator. *J Biochem* **158:** 225–234.

Okuda, K., Kato, S., Ito, T., Shiraki, S., Kawase, Y., Goto, M, *et al*. (2015b) Role of the aminotransferase domain in *Bacillus subtilis* GabR, a pyridoxal 5′-phosphate-dependent transcriptional regulator. *Mol Microbiol* **95:** 245–257.

Olmo, M.T., Sanchez-Jimenez, F., Medina, M.A., and Hayashi, H. (2002) Spectroscopic analysis of recombinant rat histidine decarboxylase. *J Biochem* **132:** 433–439.

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

Özen, A.I., and Ussery, D.W. (2012) Defining the *Pseudomonas* genus: where do we draw the line with *Azotobacter*? *Microb Ecol* **63:** 239–248.

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

Peter, H., Weil, B., Burkovski, A., Krämer, R., and Morbach, S. (1998) *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine uptake system, ProP, and the ectoine/proline/glycine betaine carrier, EctP. *J Bacteriol* **180:** 6005–6012.

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

Puig, E., Mixcoha, E., Garcia-Viloca, M., Gonzalez-Lafont, A., and Lluch, J.M. (2009) How the substrate D-glutamate drives the catalytic action of *Bacillus subtilis* glutamate racemase. *J Am Chem Soc* **131:** 3509–3521.

Rawlings, N., and Barrett, A. (2013) Introduction: metallopeptidases and their clans. In *Handbook of Proteolytic Enzymes*, 3rd ed. Elsevier, (Amsterdam; The Netherlands) pp. 325–370.

Rigali, S., Derouaux, A., Giannotta, F., and Dusart, J. (2002) Subdivision of the helix-turn-helix GntR family of bacterial

regulators in the FadR, HutC, MocR, and YtrA subfamilies. *J Biol Chem* **277:** 12507–12515.

Rivers, A.R., Smith, C.B., and Moran, M.A. (2014) An updated genome annotation for the model marine bacterium *Ruegeria pomeroyi* DSS-3. *Stand Genomic Sci* **9:** 11.

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

Roesser, M., and Müller, V. (2001) Osmoadaptation in *Bacteria* and *Archaea*: common principles and differences. *Environ Microbiol* **3:** 743–754.

Sambrook, J., Fritsch, E.F., and Maniatis, T.E. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Santini, A.C., Santos, H.R., Gross, E., and Correa, R.X. (2013) Genetic diversity of *Burkholderia* (proteobacteria) species from the caatinga and atlantic rainforest biomes in bahia, Brazil. *Genet Mol Res* **12:** 655–664.

Schröder, I., Vadas, A., Johnson, E., Lim, S., and Monbouquette, H.G. (2004) A novel archaeal alanine dehydrogenase homologous to ornithine cyclodeaminase and mu-crystallin. *J Bacteriol* **186:** 7680–7689.

Schweikhard, E.S., Kuhlmann, S.I., Kunte, H.J., Grammann, K., and Ziegler, C.M. (2010) Structure and function of the universal stress protein TeaD and its role in regulating the ectoine transporter TeaABC of *Halomonas elongata* DSM 2581(T). *Biochemistry* **49:** 2194–2204.

Schwibbert, K., Marin-Sanguino, A., Bagyan, I., Heidrich, G., Lentzen, G., Seitz, H., *et al*. (2011) A blueprint of ectoine metabolism from the genome of the industrial producer *Halomonas elongata* DSM 2581 T. *Environ Microbiol* **13:** 1973–1994.

Spies, M.A., Reese, J.G., Dodd, D., Pankow, K.L., Blanke, S.R., and Baudry, J. (2009) Determinants of catalytic power and ligand binding in glutamate racemase. *J Am Chem Soc* **131:** 5274–5284.

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

Suvorova, I. and Rodionov, D. (2016) Comparitive genomics of pyridoxal 5'-phosphate-dependent transcription factor regulons in *Bacteria*. *MGen* **2.**

Tao, Y., Shokes, J.E., McGregor, W.C., Scott, R.A., and Holz, R.C. (2012) Structural characterization of Zn(II)-, Co(II)-, and Mn(II)-loaded forms of the *argE*-encoded N-acetyl-L-ornithine deacetylase from *Escherichia coli*. *J Inorg Biochem* **111:** 157–163.

Tkaczuk, K.L.I., Shumilin A., Chruszcz, M., Evdokimova, E., Savchenko, A., and Minor, W. (2013) Structural and functional insight into the universal stress protein family. *Evol Appl* **6:** 434–449.

Todd, J.D., Kirkwood, M., Newton-Payne, S., and Johnston, A.W. (2012a) DddW, a third DMSP lyase in a model Roseobacter marine bacterium, *Ruegeria pomeroyi* DSS-3. *ISME J* **6:** 223–226.

Todd, J.D., Curson, A.R., Sullivan, M.J., Kirkwood, M., and Johnston, A.W. (2012b) The *Ruegeria pomeroyi acuI* gene has a role in DMSP catabolism and resembles *yhdH* of *E. coli* and other bacteria in conferring resistance to acrylate. *PLoS One* **7:** e35947.

Tomita, T., Miyagawa, T., Miyazaki, T., Fushinobu, S., Kuzuyama, T., and Nishiyama, M. (2009) Mechanism for multiple-substrates recognition of alpha-aminoadipate aminotransferase from *Thermus thermophilus*. *Proteins* **75:** 348–359.

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

Vermeulen, V., and Kunte, H.J. (2004) *Marinococcus halophilus* DSM 20408^T encodes two transporters for compatible solutes belonging to the betaine-carnitine-choline transporter family: identification and characterization of ectoine transporter EctM and glycine betaine transporter BetM. *Extremophiles* **8:** 175–184.

Völker, U., Engelmann, S., Maul, B., Riethdorf, S., Völker, A., Schmid, R., *et al*. (1994) Analysis of the induction of general stress proteins of *Bacillus subtilis*. *Microbiology* **140:** 741–752.

Wagner-Döbler, I., and Biebl, H. (2006) Environmental biology of the marine *Roseobacter* lineage. *Annu Rev Microbiol* **60:** 255–280.

Warren, C.R. (2014) Response of osmolytes in soil to drying and rewetting. *Soil Biol Biochem* **70:** 22–32.

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

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

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

Widderich, N., Czech, L., Elling, F.J., Könneke, M., Stöveken, N., Pittelkow, M., *et al*. (2015) Strangers in the archaeal world: osmostress-responsive biosynthesis of ectoine and hydroxyectoine by the marine thaumarchaeon *Nitrosopumilus maritimus*. *Env Microbiol* **18:** 1227–1248.

Widderich, N., Kobus, S., Höppner, A., Ricela, R., Seubert, A., Dickschat, J.S., *et al*. (2016) Biochemistry and crystal structure of the ectoine synthase: an iron-containing member of the cupin superfamily. *PLoS One* **11:** e0151285.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Identification of osmostress protectants for *R. pomeroyi*. Growth of the *R. pomeroyi* strain J470 in basal minimal medium containing 1 M NaCl in the presence of different compatible solutes (1 mM) is shown. The growth

yielded of the cultures was recorded after their incubation for 48 h at 30°C. A reference culture of *R. pomeroyi* strain J470 grown without the addition of a compatible solute to the growth medium is marked in grey. Red bars indicate an osmoprotective effect of the compatible solute added. Growth of cultures marked in black bars shows no osmoprotection by the added compatible solute.

Fig. S2. Transcriptional analyses of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* gene cluster (A) Genetic map of the *enuR-uehABC-usp-eutABCDE-asnC-ssd-atf* genes in the genome of *R. pomeroyi* DSS-3. (B) Northern Dot Blot analyses of the level of transcription of the *R. pomeroyi enuR*, *uehA*, *ssd*, *atf* and *dddW* genes. Total RNA was prepared from *R. pomeroyi* DSS-3 cells grown in basal medium containing either glucose, ectoine, or 5-hydroxyectoine as the sole carbon source and was spotted at different concentrations (3 μg - 0.18 μg) onto a nylon membrane. The RNA was then hybridized to DIG-labeled anti-sense RNA transcripts prepared in vitro for the specific genes under study. The transcript of the DMSP lyase gene (*dddW*) from *R. pomeroyi* DSS-3, a gene involved in DMSP catabolism (Todd et al., 2012a), was used as a control.

Fig. S3. Growth curves of *R. pomeroyi* cultures in basal media containing glucose (28 mM) as a carbon source and NH_4Cl as a nitrogen source. Shown are *R. pomeroyi* strains J470 (wild type), ASR12 [Δ*(eutABCDE-asnC-ssd-atf::Gm*)1], and ASR6 [Δ(*enuR-uehABC-usp-eutABCDE-asnC-ssd-atf::Gm*)1].

Fig. S4. Detailed description of the EutABC-mediated conversion of 5-hydroxyectoine to ectoine. The reaction conducted by each of these enzymes is depicted and described in detail.

Fig. S5. Model of the EnuR regulatory protein. (A) The crystal structure of the *Bacillus subtilis* GabR protein in complex with PLP (protein database accession code 4N0B) (Edayathumangalam et al., 2013) was chosen as a template to generate an *in silico* model of the transcriptional regulator EnuR. For the visualization of this structure the PyMol suit (https://www.pymol.org/) was used. The helix-turn-helix DNA binding motif of the modelled EnuR protein is depicted in yellow. The C-terminal aminotransferase I-like domain of EnuR is shown in grey. (B) The PLP cofactor (shown as sticks) covalently attached to Lys-302 was modelled into the structure using an overlay of the EnuR model and the GabR template.

Fig. S6. Assessment of the quaternary assembly of the EnuR-*Strep*-tag II protein and its Lys302/His mutant (EnuR*-*Strep*-tag II). Recombinant EnuR-*Strep*-tag II (A) and EnuR*-*Strep*-tag II (B) were purified by affinity chromatography on a streptactin matrix and analyzed by SEC. In the inserts, the chromatographic behavior of the EnuR-*Strep*-tag II and EnuR*-*Strep*-tag II proteins (approximately 103 kDa) (red dots) is shown relative that that of the marker proteins, carbonic anhydrase from bovine erythrocytes (29 kDa), albumine from bovine serum (66 kDa), and alcohol dehydrogenase from yeast (150 kDa) (black dots).

Fig. S7. Taxonomic distribution of 5-hydroxyectoine/ectoine uptake and catabolic genes. An alignment of 539 amino acid sequences homologous to the EutD protein from *Ruegeria pomeroyi* DSS-3 was used to construct a phylogenetic tree. Sequences were identified through a BLAST search on the JGI web server and aligned using ClustalΩ. The phylogenetic tree was constructed using the iTOL web-server (Letunic and Bork, 2011). The color code outlines the distribution of EutD among the classes of the *Proteobacteria*. The presence of a transporter in the direct vicinity of the predicted *eutD* gene cluster is indicated by an orange (ABC-type), or dark blue (TRAP-type), circle. A green circle indicates the presence of a TeaABC-type TRAP transporter. Pink pentagons are indicating an association of an *enuR*-type *regulatory* gene with the *eutD* gene cluster. The presence of 5-hydroxyectoine degradation genes is depicted using green (presence of *eutABC* genes) or red (presence of only *eutBC* genes) squares. The presence of genes that were not found in the *eutD* gene clusters derived from *S. meliloti* or *R. pomeroyi* are presented in pink (*argE*), light blue (NADPH:quinone reductase), or green (γ-glutamyltransferase) circles. The presence of an *ectABC* gene cluster (ectoine synthesis genes) is indicated by green triangles; the presence of an *ectD* gene (ectoine hydroxylase) in the respective genome sequences is marked by an orange triangle.

Fig. S8. Amino acid sequence alignment of the 5-hydroxyectoine/ectoine ligand-binding protein (EhuB) from the EhuABCD ABC transporter from *S. meliloti* with selected EhuB-type solute receptor proteins from predicted 5-hydroxyectoine/ectoine catabolizing bacteria. The red arrowheads mark those amino acid residues involved in ligand binding (Hanekop et al., 2007).

Fig. S9. Amino acid sequence alignment of the 5-hydroxyectoine/ectoine ligand-binding protein (UehA) from the UehABC TRAP transporter from *R. pomeroyi DSS-3* with selected UehA-type solute receptor proteins from predicted 5-hydroxyectoine/ectoine-catabolizing bacteria. The red arrowheads mark those amino acid residues involved in ligand binding (Lecher et al., 2009).

Fig. S10. Amino acid sequence alignment of the 5-hydroxyectoine/ectoine ligand-binding protein (TeaA) from the TeaABC TRAP transporter from *H. elongata* with selected TeaA-type solute receptor proteins from predicted 5-hydroxyectoine/ectoine-catabolizing bacteria. The red arrowheads mark those amino acid residues involved in ligand binding (Kuhlmann et al., 2008).

Table S1. Bacterial strains and plasmids used in this study.

Table S2. DNA primers used in this study.