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Transcriptional regulation of ectoine catabolism in response to multiple metabolic and environmental cues

Annina Schulz,[1] Lucas Hermann,[1] Sven-Andreas Freibert,[2] Tobias Bönig,[1] Tamara Hoffmann,[1] Ramona Riclea,[3,4] Jeroen S. Dickschat,[3,4] Johann Heider[1,5] and Erhard Bremer[1,5]*

[1]*Department of Biology, Laboratory for Microbiology, Philipps-University Marburg, Karl-von-Frisch-Str. 8, D-35043 Marburg, Germany.*

[2]*Department of Medicine, Institute for Cytobiology and Cytopathology, Philipps-University Marburg, Robert-Koch Str. 6, D-35032 Marburg, Germany.*

[3]*Institute of Organic Chemistry, Technical University Braunschweig, D-38106 Braunschweig, Germany.*

[4]*Kekulé-Institute for Organic Chemistry and Biochemistry, Friedrich-Wilhelms-Universität Bonn, D-53121 Bonn, Germany.*

[5]*LOEWE-Center for Synthetic Microbiology, Philipps-University Marburg, Hans-Meerwein Str. 6, D-35043 Marburg, Germany.*

Summary

Ectoine and hydroxyectoine are effective microbial osmostress protectants, but can also serve as versatile nutrients for bacteria. We have studied the genetic regulation of ectoine and hydroxyectoine import and catabolism in the marine *Roseobacter* species *Ruegeria pomeroyi* and identified three transcriptional regulators involved in these processes: the GabR/MocR-type repressor EnuR, the feast and famine-type regulator AsnC and the two-component system NtrYX. The corresponding genes are widely associated with ectoine and hydroxyectoine uptake and catabolic gene clusters (*enuR*, *asnC*), and with microorganisms predicted to consume ectoines (*ntrYX*). EnuR contains a covalently bound pyridoxal-5'-phosphate as a co-factor and the chemistry underlying the functioning of MocR/GabR-type regulators typically requires a system-specific low molecular mass effector molecule. Through ligand binding studies with purified EnuR, we identified *N*-(alpha)-L-acetyl-2,4-diaminobutyric acid and L-2,4-diaminobutyric acid as inducers for EnuR that are generated through ectoine catabolism. AsnC/Lrp-type proteins can wrap DNA into nucleosome-like structures, and we found that the *asnC* gene was essential for use of ectoines as nutrients. Furthermore, we discovered through transposon mutagenesis that the NtrYX two-component system is required for their catabolism. Database searches suggest that our findings have important ramifications for an understanding of the molecular biology of most microbial consumers of ectoines.

Received 25 June, 2017; revised 29 August, 2017; accepted 31 August, 2017. *For correspondence. E-mail: bremer@staff.uni-marburg.de; Tel. (+49)-6421-2821529; Fax (+49)-6421-2828979.

Introduction

Ectoine and its derivative 5-hydroxyectoine (Pastor *et al.*, 2010; Kunte *et al.*, 2014) are members of a selected group of organic osmolytes, the compatible solutes. Many *Bacteria* and some *Archaea* use these types of compounds to fend off the detrimental effects of high osmolarity/salinity on cellular physiology and growth (Kempf and Bremer, 1998; Roesser and Müller, 2001). Their accumulation promotes the hydration of the cytoplasm under osmotically unfavourable environmental conditions, and thereby allows maintaining vital turgor within physiologically acceptable boundaries (Bremer and Krämer, 2000; Wood, 2011; Booth, 2014). The physicochemical attributes of ectoines make them highly compliant with cellular physiology, biochemistry and the functionality of macromolecular structures and assemblies (Lippert and Galinski, 1992; Manzanera *et al.*, 2002; Harishchandra *et al.*, 2010; Tanne *et al.*, 2014; Zaccai *et al.*, 2016). As a result, these compounds can be accumulated by high osmolarity challenged cells to exceedingly high cellular levels, either through transport or synthesis (Kuhlmann and Bremer, 2002; Kuhlmann *et al.*, 2011). The ability to synthesize ectoines as osmotic stress protectants is an ecophysiologically important trait for many microorganisms that populate marine, terrestrial, or plant-associated habitats (Widderich *et al.*, 2014; 2016b). The biosynthetic routes for ectoine

and hydroxyectoine are genetically and biochemically rather well understood. They rely on the EctABC enzymes to produce ectoine from aspartate-β-semialdehyde, a central intermediate in amino acid metabolism and cell wall synthesis, and on the EctD enzyme to further convert it to 5-hydroxyectoine (Pastor *et al.*, 2010; Kunte *et al.*, 2014) (Fig. 1).

Ectoines also serve as carbon, nitrogen and energy sources for different microbial species (Galinski and Herzog, 1990; Manzanera *et al.*, 2002; Jebbar *et al.*, 2005; Vargas *et al.*, 2006; Rodriguez-Moya *et al.*, 2010; Schwibbert *et al.*, 2011; Schulz *et al.*, 2017). These compounds are released into the environment from producer microorganisms either through the transient opening of mechanosensitive channels as a consequence of osmotic down-shocks, through secretion, or on cellular decomposition (Welsh, 2000; Grammann *et al.*, 2002; Booth, 2014; Widderich *et al.*, 2016b). Given the wide occurrence of microorganisms capable of synthesizing ectoines (Widderich *et al.*, 2014; 2016b), it does not come as a surprise that these compounds have been detected in different ecosystems (Mosier *et al.*, 2013; Warren, 2014; 2016).

In contrast to the wide taxonomic affiliation of hydroxyectoine/ectoine producers (Widderich *et al.*, 2014; 2016b), all currently known, or predicted, microbial consumers of ectoines are members of the *Proteobacteria* (Schulz *et al.*, 2017). Building on previous data (Jebbar *et al.*, 2005; Schwibbert *et al.*, 2011), we have recently proposed a pathway for the complete route of hydroxyectoine/ectoine uptake and catabolism (Schulz *et al.*, 2017) (Fig. 1) in the marine bacterium *Ruegeria pomeroyi* DSS-3 (Moran *et al.*, 2004), a member of the widely distributed and metabolically versatile *Roseobacter* clade (Wagner-Döbler and Biebl, 2006; Luo and Moran, 2014; Simon *et al.*, 2017). In this bacterium, import of ectoines is mediated by a high affinity binding-protein-dependent and substrate-inducible TRAP-type transport system (UehABC) (Lecher *et al.*, 2009; Mulligan *et al.*, 2011) (Fig. 1). Once imported, the catabolism of ectoines can be broken down to three functional modules: (i) three enzymes (EutABC) convert hydroxyectoine to ectoine, (ii) the ectoine ring is then hydrolytically opened by the ectoine hydrolase EutD to form *N*-(α)-L-acetyl-2,4-diaminobutyric acid [*N*-(α)-ADABA] and (iii) this intermediate is subsequently further metabolized to L-aspartate via the EutE-Atf-Ssd enzymes (Schulz *et al.*, 2017) (Fig. 1).

The hydroxyectoine/ectoine uptake and catabolic genes of *R. pomeroyi* DSS-3 are genetically organized in a 13.5-Kbp operon whose expression is mediated by two promoters, one of which is located upstream of *enuR* and the other is present in front of *uehA*. The *enuR* gene encodes a major regulatory gene for the hydroxyectoine/ectoine uptake and catabolic gene cluster (Schulz *et al.*, 2017) (Fig. 1). Its promoter operates constitutively at a low transcriptional level, whereas the promoter positioned upstream of *uehA* directs the expression of the transport and catabolic genes; its transcriptional activity is strongly enhanced by the presence of ectoines in the growth medium. As a result, a robust substrate induction of hydroxyectoine/ectoine uptake and catabolic activities is observed in *R. pomeroyi* DSS-3 (Lecher *et al.*, 2009; Schulz *et al.*, 2017).

A bioinformatics assessment of the genome context of the hydroxyectoine/ectoine catabolic gene clusters in a substantial number of bacteria (Schulz *et al.*, 2017) revealed a strong correlation with a gene coding for a member of the GabR/MocR family of regulatory proteins, a sub-group of the GntR super-family of transcriptional regulators (Rigali *et al.*, 2002; Bramucci *et al.*, 2011; Suvorova and Rodionov, 2016). The name chosen by us for this gene stands for **e**ctoine **n**utrient **u**tilization **r**egulator (*enuR*) but orthologues of the same type of gene have been referred to in the literature either as *ehuR* or as *eutR* (Suvorova and Rodionov, 2016; Yu *et al.*, 2017). EnuR serves as a repressor of the hydroxyectoine/ectoine uptake and catabolic genes, both in *R. pomeroyi* DSS-3 (Schulz *et al.*, 2017) and in *Sinorhizobium meliloti* (Yu *et al.*, 2017), a plant-root associated bacterium in which molecular details of ectoine import and catabolism were initially described through a proteomic approach and crystallographic analysis of the ligand binding protein of a hydroxyectoine/ectoine-specific ABC-type importer (EhuABCD) (Jebbar *et al.*, 2005; Lecher *et al.*, 2009).

MocR/GabR-type regulators are widely distributed in Gram-positive and Gram-negative bacteria but only a few of them have been functionally characterized (Belitsky and Sonenshein, 2002; Wiethaus *et al.*, 2008; Belitsky, 2014; Okuda *et al.*, 2015b; Takenaka *et al.*, 2015; Tramonti *et al.*, 2017). They possess a conserved structural organization with an N-terminal DNA-reading head containing a winged helix-turn-helix DNA-binding motif that is connected via a flexible linker region to a large carboxy-terminal effector-binding/dimerization domain. This latter domain is structurally related to aminotransferases of type-I fold (Edayathumangalam *et al.*, 2013; Milano *et al.*, 2015; Suvorova and Rodionov, 2016). It frequently contains a covalently bound pyridoxal-5'-phosphate (PLP) co-factor (attached to a Lys-residue) but MocR/GabR-type regulators do not perform a full aminotransferase enzyme reaction (Edayathumangalam *et al.*, 2013; Okuda *et al.*, 2015a,b; Takenaka *et al.*, 2015; Park *et al.*, 2017; Wu *et al.*, 2017). Instead, a partial aminotransferase reaction occurs that initially involves the covalent binding of a system-specific low molecular mass effector molecule to the protein-bound PLP co-factor (the internal aldimine) and the subsequent formation of an external aldimine between PLP and the effector molecule. These chemical reactions are transduced into a conformational change of the entire

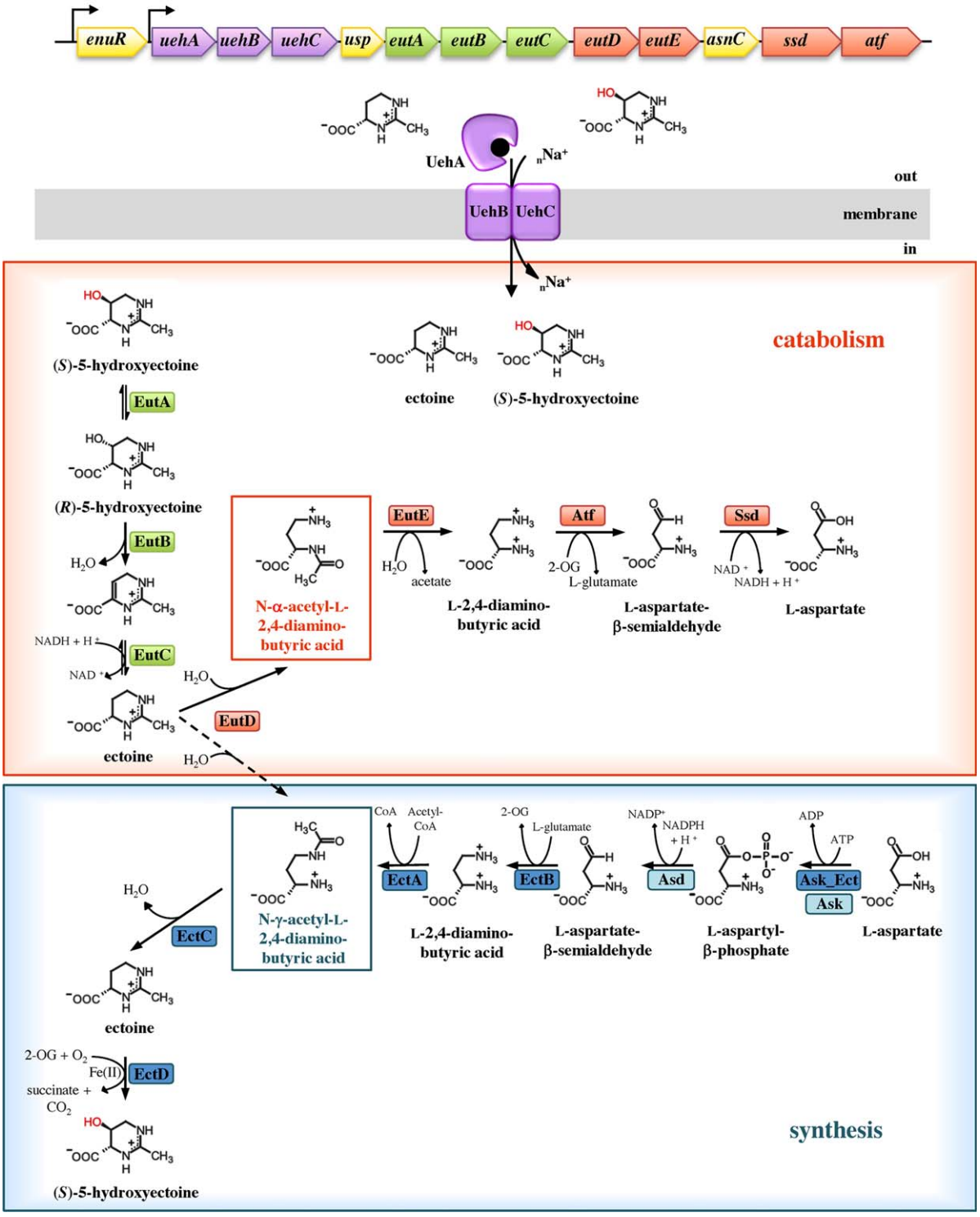

Fig. 1. Anabolic and catabolic routes for ectoines. The genetic organization of the hydroxyectoine/ectoine catabolic gene cluster present in the *R. pomeroyi* DSS-3 genome is shown (Moran *et al.*, 2004; Schulz *et al.*, 2017). The sketches for the synthesis route for ectoine and hydroxyectoine (Ono *et al.*, 1999; Bursy *et al.*, 2007; Stöveken *et al.*, 2011) and that for their catabolism (Schwibbert *et al.*, 2011; Schulz *et al.*, 2017) are based on previously published data. All enzymes involved in ectoine and hydroxyectoine biosynthesis have been enzymatically studied (Ono *et al.*, 1999; Bursy *et al.*, 2007; Stöveken *et al.*, 2011), while EutD (DeoA) is the only enzyme from the catabolic route whose function has been experimentally assessed. The information on the generation of both *N*-(α)-ADABA and *N*-(γ)-ADABA by the recombinant EutD (DoeA) enzyme from *H. elongata* is based on data reported by Schwibbert *et al.* (Schwibbert *et al.*, 2011).

regulatory protein, which then dictates the DNA-binding and functional properties of MocR/GabR-type proteins to function either as activators or repressors (or both) of gene transcription (Belitsky and Sonenshein, 2002; Wiethaus *et al.*, 2008; Edayathumangalam *et al.*, 2013; Okuda *et al.*, 2015a,b; Takenaka *et al.*, 2015; Park *et al.*, 2017; Tramonti *et al.*, 2017; Wu *et al.*, 2017). EnuR possesses such a covalently bound PLP molecule, and Lys-302 present in its carboxy-terminal aminotransferase domain has been identified through *in silico* modelling and mutant studies as the residue to which the co-factor is attached (Schulz *et al.*, 2017).

Although an exogenous supply of ectoines triggers a strong induction of the transcription of the hydroxyectoine/ectoine uptake and catabolic genes in *R. pomeroyi* DSS-3 (Lecher *et al.*, 2009; Schulz *et al.*, 2017), they cannot be the true inducer molecules because the chemistry underlying the interaction of the system-specific effector molecule with the Lys-bound PLP co-factor requires a primary amino group (Bramucci *et al.*, 2011; Edayathumangalam *et al.*, 2013; Okuda *et al.*, 2015a,b; Takenaka *et al.*, 2015; Suvorova and Rodionov, 2016; Park *et al.*, 2017; Wu *et al.*, 2017). Since free amino groups are only generated after the first step of ectoine degradation (Fig. 1), it seemed plausible that catabolic intermediates would function as internal inducer(s) for the EnuR repressor protein. Indeed, based on DNA binding studies with the EnuR orthologue (EhuR) from *S. meliloti* (Jebbar *et al.*, 2005), 2,4-diaminobutyric acid (DABA) (Fig. 1) has been proposed as a potential inducer for hydroxyectoine/ectoine uptake and utilization genes but direct binding studies between EhuR and DABA have not been reported (Yu *et al.*, 2017).

Since DABA is also produced in microbial pathways not involved in ectoine catabolism (Ikai and Yamamoto, 1997; Du *et al.*, 2013; Fidalgo *et al.*, 2016), we set out to genetically and biochemically define the physiologically most relevant system-specific inducer for EnuR. We identify it here as *N*-(α)-ADABA, a highly specific intermediate of ectoine catabolism (Schwibbert *et al.*, 2011; Schulz *et al.*, 2017) (Fig. 1). The hydroxyectoine/ectoine import and catabolic gene cluster of *R. pomeroyi* DSS-3 contains a gene (*asnC*) (Fig. 1) encoding a member of the *feast-and-famine* class of transcriptional regulators (Yokoyama *et al.*, 2006). We found that the AsnC protein is essential for the ability of *R. pomeroyi* DSS-3 to use ectoine as a carbon source. Finally, the tetrahydropyrimidines ectoine and hydroxyectoine contain two nitrogen atoms (Fig. 1), and thus are particularly valuable compounds in nutrient-deprived ecosystems (Lidbury *et al.*, 2014; 2015; Taubert *et al.*, 2017). We discovered through transposon mutagenesis that the two-component NtrYX regulatory system (Fernandez *et al.*, 2017) is a key player in the use of ectoines as nutrients by *R. pomeroyi* DSS-3. In conjunction with extensive database searches, the physiological and molecular data reported here for the model system *R. pomeroyi* DSS-3 have important implications for an understanding of the consumption of ectoines in general, and paint a rather complex picture of the genetic control of this ecophysiologically relevant catabolic process.

Results

Ectoine- and hydroxyectoine-mediated induction of import and catabolic genes

To study the expression of the hydroxyectoine/ectoine uptake and catabolic genes (Fig. 1), we used three previously constructed transcriptional *lacZ* reporter fusions (Schulz *et al.*, 2017). Plasmid pBAS19 carries the promoter present in front of *enuR*, while plasmid pBAS21 carries the promoter present in front of the *uehA* transporter gene. Plasmid pBAS20 carries the same *uehA-lacZ* operon fusion as plasmid pBAS21 but harbours, in addition, an intact *enuR* gene that is expressed from its native promoter (Fig. 2A). These three reporter plasmids were introduced via conjugation into the *R. pomeroyi* strain J470 (Supporting Information Table S1), a rifampicin-resistant derivative of the wild-type isolate DSS-3 (Moran *et al.*, 2004; Todd *et al.*, 2012) and used them to evaluate the influence of an external supply of ectoines on the transcriptional activity of the *enuR* and *uehA* promoters. Growth of the cells in a glucose and ammonium based minimal medium resulted only in very low expression levels (approximately 20 Miller Units) of either the *enuR-lacZ* and *uehA-lacZ* reporter fusions (Fig. 2B). We observed that the promoter in front of *enuR* was not responsive to the presence of ectoines, while the transcriptional activity of the *uehA* promoter was strongly substrate inducible (Fig. 2B). Ectoine induced the expression of the *uehA-lacZ* reporter fusion present on plasmid pBAS21 30-fold, and adding hydroxyectoine to the growth medium led to a 64-fold increased expression level. The corresponding values for the induction of *uehA-lacZ* gene expression in a strain carrying the reporter plasmid pBAS20 (*enuR*$^+$) are 41-fold (for ectoine) and 95-fold (for hydroxyectoine), respectively (Fig. 2B). Hence, hydroxyectoine seems to be a more potent inducer of *uehA-lacZ* transcription. This phenomenon was particularly notable when these cells carried the *enuR*$^+$ plasmid pBAS20 (Fig. 2B).

We, and others, have recently shown that EnuR (= EhuR) serves as a repressor for ectoine catabolism (Schulz *et al.*, 2017; Yu *et al.*, 2017). Therefore, it came somewhat as a surprise that the *uehA-lacZ* reporter fusion present on the *enuR*$^+$ plasmid pBAS20 exhibited higher induction levels in response to ectoines in comparison with plasmid pBAS21 that lacks an intact *enuR* regulatory gene (Fig. 2A and B). Furthermore, *R. pomeroyi* J470 that was used as a background strain for the *lacZ* reporter fusion experiments carries itself an intact chromosomal copy of

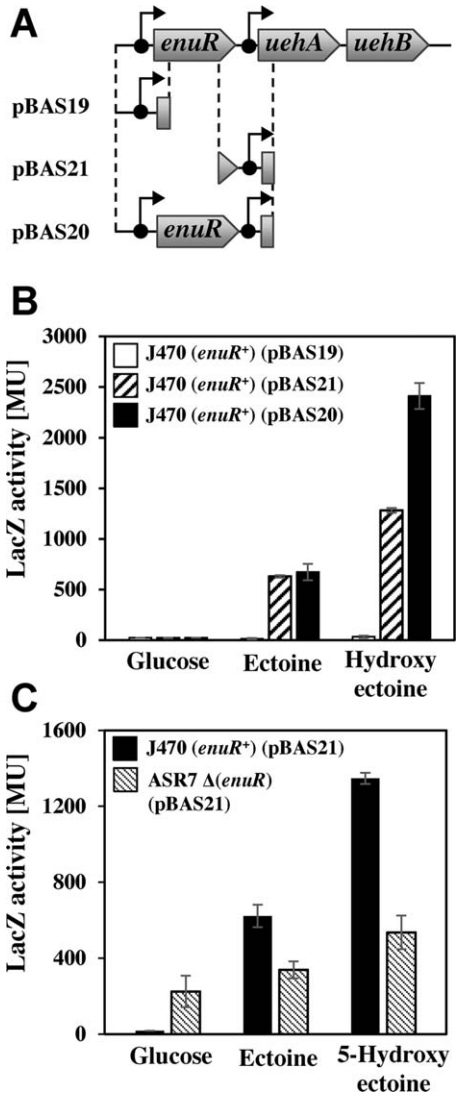

Fig. 2. Substrate induction of *uehA-lacZ* expression and role of EnuR. (A) Genetic make-up of the DNA fragments used to construct *enuR-lacZ* and *uehA-lacZ* gene fusions using the *lacZ* reporter plasmid pBIO1878 (Todd *et al*., 2012). The approximate positions of the so-far unidentified promoters present in front of *enuR* and *uehA* (Schulz *et al*., 2017) are indicated by the arrows. (B) Transcriptional activity of *enuR-lacZ* and *uehA-lacZ* reporter fusions in response to ectoine or hydroxyectoine availability in the growth medium in the *enuR*$^+$ wild-type strain J470, a Rif^R-derivative of *R. pomeroyi* DSS3. (C) Comparison of *uehA-lacZ* reporter fusion activity in the wild-type strain J470 and its Δ(*enuR*) mutant derivative strain ASR7.

the *enuR* gene. Hence, either EnuR possesses both repressor and activator functions as previously reported for GabR (Belitsky and Sonenshein, 2002; Belitsky, 2004; Edayathumangalam *et al*., 2013), or alternatively, DNA-sequences located upstream of *uehA* required for full genetic control of the *uehA* promoter were removed during construction of plasmid pBAS21 (Fig. 2A).

We also assessed the influence of the chromosomal *enuR* gene on the activity of the *uehA-lacZ* reporter construct present on plasmid pBAS21 that itself does not harbour an intact *enuR* gene (Fig. 2A). When the *enuR* gene was disrupted (strain ASR7) in the chromosome of *R. pomeroyi*, the activity of the *uehA-lacZ* reporter fusion was high in cells grown in the absence of ectoines, a 13-fold increase over its isogenic *enuR*$^+$ parent strain J470 grown in the same glucose- and ammonium-based basal minimal medium (Fig. 2C). This is consistent with the notion that EnuR acts genetically as a repressor (Schulz *et al*., 2017; Yu *et al*., 2017). Induction of the reporter fusion by either ectoine or hydroxyectoine in a chromosomal *enuR* deletion strain was less prominent, but was not completely eliminated (Fig. 2C). This latter finding indicates that in addition to EnuR, other regulatory proteins might contribute to substrate induction of the *uehA-lacZ* reporter fusion expression.

Substrate-mediated induction of ectoine catabolism depends on a PLP co-factor covalently bound to EnuR

When EnuR is produced in *E. coli* as a recombinant protein, the affinity-purified EnuR has a strong yellow colour and possesses spectroscopic properties typical for PLP-containing proteins (Phillips, 2015; Schulz *et al*., 2017). The substitution of Lys-302, the residue to which the PLP molecule is attached, with a His residue resulted in a mutant protein (K302/H; EnuR*) that had lost its yellow colour and the characteristic spectroscopic properties of the wild-type protein (Schulz *et al*., 2017). We tested the influence of the K302/H amino acid substitution mutation on the regulatory properties of EnuR to assess the role played by the covalently attached PLP molecule for the substrate-mediated induction of the hydroxyectoine/ectoine transport and catabolic gene cluster.

To this end, we introduced the wild-type *enuR* gene (present on plasmid pBAS20) and its mutant *enuR** derivative (present on plasmid pBAS23) (Fig. 3A) into *R. pomeroyi* strains that either lacked or carried an intact chromosomal copy of the *enuR* gene. The *enuR*$^+$ gene present on pBAS20 was able to repress the elevated level of *uehA-lacZ* transcription observed in *enuR* mutant cells grown with glucose and ammonium (Fig. 2C) and allowed induction of promoter activity in response to the availability of ectoines (Fig. 3B). Not surprisingly, the same pattern of transcription was found in a strain carrying an additional intact chromosomal copy of *enuR*. In contrast, the presence of the *enuR** mutation on plasmid pBAS23 no longer allowed substrate induction, regardless of whether the chromosomal *enuR* gene was intact or not (Fig. 3B). The EnuR* protein thus behaves genetically as a dominant negative repressor and, unlike its EnuR wild-type counterpart, was unable to respond to the availability of ectoines

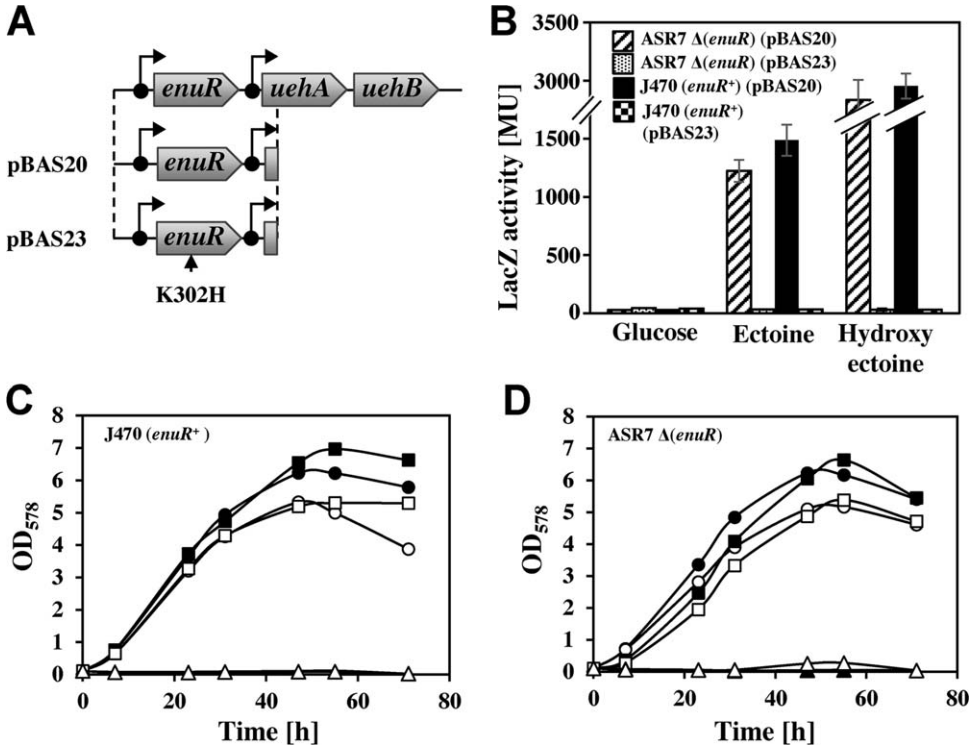

Fig. 3. Substrate induction of *uehA-lacZ* expression and the role of the mutant EnuR* protein on *uehA-lacZ* expression. (A) Genetic make-up of the DNA-fragments used to construct *uehA-lacZ* gene fusions using the *lacZ* reporter plasmid pBIO1878. Plasmid pBAS20 carries the wild-type *enuR* gene, whereas plasmid pBAS23 carries the Lys-302 to His *enuR** substitution mutation. (B) Transcriptional activity of *uehA-lacZ* reporter fusions in response to ectoine or hydroxyectoine availability in the growth medium in the wild-type strain J470 (*enuR*$^+$) or its Δ(*enuR*) mutant derivative strain ASR7 carrying plasmids with either an intact (pBAS20) or mutant (*enuR**) (pBAS23) *enuR* gene. (C) Growth of the *enuR*$^+$ *R. pomeroyi* wild-type strain J472 in basal minimal medium with either ectoine (black symbols) or hydroxyectoine (open symbols) carrying either the empty fusion vector pBIO1878 (open and closed circles), the *enuR*$^+$ plasmid pBAS20 (open and closed squares), or the *enuR** plasmid (open and closed triangles). (D) The same growth conditions and plasmid-bearing strains were used as described in (C), except that the genetic background of the host strain ASR7 carries a chromosomal Δ(*enuR*) mutation.

in the growth medium (Fig. 3B). The properties of EnuR* to act as a dominant negative repressor were also manifested when we analysed the effects of the *enuR** mutation on the growth of both *enuR*$^+$ and *enuR* mutant *R. pomeroyi* cells harbouring either plasmid pBAS20 (*enuR*$^+$) or pBAS23 (*enuR**). In both genetic backgrounds, use of ectoines as joint carbon and nitrogen sources was no longer possible when the cells carried pBAS23 (*enuR**) while the presence of the *enuR*$^+$ plasmid pBAS20 permitted growth of *R. pomeroyi* cultures to high optical densities (OD_{574} values between approximately 5 and 7) (Fig. 3C and D).

Identification of internal inducers of the EnuR regulatory protein

MocR/GabR-type regulators (Suvorova and Rodionov, 2016) that possess a covalently attached PLP molecule depend on a system-specific effector molecule to alter their regulatory properties; e.g., GABA for GabR, taurine (or derivatives of it) for TauR, and a dipeptide for DdlR (Wiethaus *et al.*, 2008; Takenaka *et al.*, 2015; Park *et al.*, 2017; Wu *et al.*, 2017). We set out to identify the system-specific effector molecule for EnuR. To detect and quantify ligand binding by the purified EnuR protein, we used microscale thermophoresis (MST), a method that traces the movement of fluorescently labelled proteins in a temperature gradient in response to the presence of a ligand (Duhr and Braun, 2006; Wienken *et al.*, 2010).

The chemistry underlying the formation of the internal and external aldimine depends on the presence of a primary amino group in the system-specific effector molecule (Takenaka *et al.*, 2015; Suvorova and Rodionov, 2016; Park *et al.*, 2017; Wu *et al.*, 2017). Neither ectoine nor hydroxyectoine possess such a primary amino group (Fig. 1) and consequently, neither one of them bound to the purified EnuR protein when we tested concentrations up to 10 mM in MST experiments (Supporting Information Fig. S1). Inspection of the hydroxyectoine/ectoine catabolic route revealed that the cleavage of the ectoine ring by the EutD hydrolase generates *N*-(α)-acetyl-2,4-diaminobutyric acid [*N*-(α)-ADABA], the first metabolite possessing a

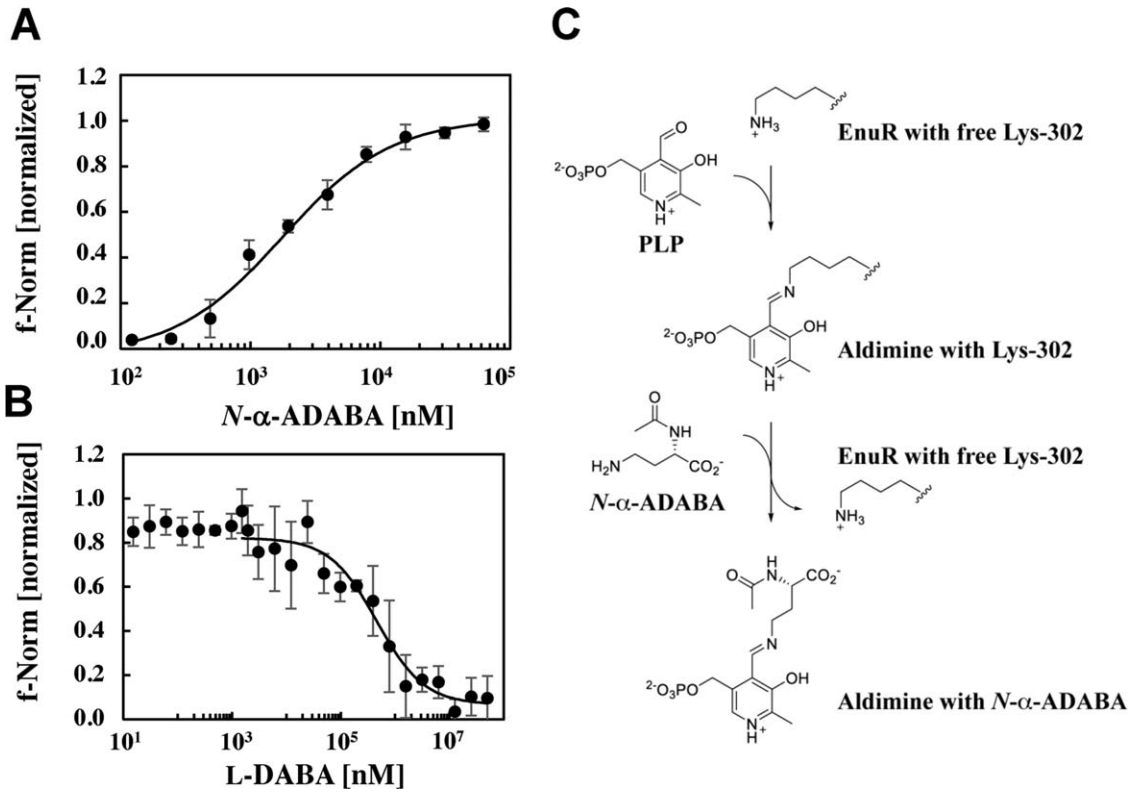

Fig. 4. Ligand binding by the wild-type EnuR protein. Purified EnuR protein (200 nM) was incubated with increasing concentrations of (A) *N*-(α)-ADABA, or (B) DABA, and the ability of these compounds to bind to the EnuR was assessed by microscale thermophoresis. (C) Proposal for the PLP-dependent and *N*-(α)-ADABA-triggered formation of initially the internal and subsequently the external aldimine in EnuR. PLP is initially bound in this sequence of chemical reactions by the side-chain of Lys-302 present in the carboxy-terminal aminotransferase domain of EnuR (Schulz *et al.*, 2017). This scheme was adapted from data reported for the MocR/GabR-type regulators GabR and DdlR that respond to the availability of GABA and a di-peptide (D-alanyl-D-alanine), respectively (Edayathumangalam *et al.*, 2013; Okuda *et al.*, 2015a,b; Takenaka *et al.*, 2015; Park *et al.*, 2017; Wu *et al.*, 2017).

primary amino group (Fig. 1). To the best of our knowledge, *N*-(α)-ADABA is exclusively generated during ectoine catabolism, making this compound an interesting candidate as system-specific effector molecule for EnuR. Indeed, ligand-binding measurements in MST experiments revealed strong binding of this metabolite by EnuR with a K_d of 1.7 ± 0.3 μM (Fig. 4A). Importantly, the EnuR* mutant protein was unable to bind *N*-(α)-ADABA, even at a concentration of 1 mM (Supporting Information Fig. S1).

The isomer of the high affinity EnuR ligand *N*-(α)-ADABA, *N*-(γ)-ADABA, is an intermediate in ectoine biosynthesis (Fig. 1) and serves as the main substrate for the ectoine synthase (EctC) (Ono *et al.*, 1999; Widderich *et al.*, 2016a). In some ectoine consumers (e.g., *Halomonas elongata*), *N*-(γ)-ADABA can apparently also be generated through the ectoine hydrolase (EutD/DoeA) as a by-product of the main chemical reaction (Fig. 1) (Schwibbert *et al.*, 2011). However, it is not clear so far whether this is only the case in the sub-group of hydroxyectoine/ectoine consumers that also synthesize ectoines as osmostress protectants; e.g., *H. elongata* (Schulz *et al.*, 2017). Given the close chemical relatedness of *N*-(α)-ADABA and *N*-(γ)-ADABA (Fig. 1), we also tested binding of *N*-(γ)-ADABA to EnuR by MST but detected no binding up to the highest concentration (1 mM) tested (Supporting Information Fig. S1).

Another intermediate of ectoine catabolism is L-2,4-diaminobutyric acid (DABA) (Fig. 1) which has been suggested to function as an inducer for the EnuR orthologue EhuR from *S. meliloti* (Yu *et al.*, 2017). We found that DABA is also recognized by the *R. pomeroyi* EnuR protein and determined a K_d of ligand binding of 457 ± 1 μM via MST (Fig. 4B), an approximately 230-fold reduction in ligand binding with respect to *N*-(α)-ADABA. Taken together, these ligand binding data identify *N*-(α)-ADABA (highly efficient) and DABA (moderately effective) as internal inducers for the EnuR regulatory protein, both of which are generated during ectoine catabolism (Fig. 1).

In silico and experimental analysis of the uehA regulatory region

Using comparative genomics and metabolic reconstruction, Suvorova and Rodionov (2016) recently analysed the

distribution of MocR/GabR-type regulators in a non-redundant set of 390 microbial genomes representing 43 diverse lineages of *Bacteria*. They predicted the putative operator binding sites of various sub-groups of this family of transcriptional regulators through bioinformatics. These authors also focused on EnuR-type proteins (referred to in their study as EutR) and suggested that they recognize a conserved inverted repeat DNA sequence as their operator sequences DNA-binding motif (Suvorova and Rodionov, 2016). We searched the 433 bp DNA-fragment (Fig. 5A) carried by the EnuR-responsive *uehA-lacZ* operon fusion plasmid pBAS21 for the presence of DNA sequences resembling the EutR consensus sequence proposed by Suvorova and Rodionov (2016). We found two possible operator binding sites for EnuR in the 97 bp intergenic region between the 3'-end of the *enuR* gene and the 5'-end of *uehA* (Fig. 5A). One of these putative operators (16 bp) adheres closely to the proposed consensus sequence. The five outermost base-pairs in each of the corresponding inverted repeat sequences match perfectly to the most conserved base-pairs in the consensus EutR-type operator sequence proposed by Suvorova and Rodionov (2016) by inspecting 69 microbial genome sequences (Fig. 5B). Six base-pairs downstream of this putative EnuR operator site, a second copy of a sequence resembling the consensus operator proposed by Suvorova and Rodionov (2016) is present, but it is two base-pairs longer (Fig. 5A and B). This second putative operator sequence contains an inverted repeat as well, where the five-outermost base-pairs in each of the inverted repeat structures match again perfectly to the most conserved base-pairs in the EutR consensus sequence (Fig. 5B).

DNase I footprinting analysis conducted to determine the binding site of the EnuR orthologue (EhuR) of the *S. meliloti ehuABCD-eutABCDE* promoter have also identified two operator sequences (Fig. 5B), one of which overlaps the putative −35 region of the promoter present in front of the *S. meliloti ehuA* gene (Yu *et al.*, 2017). These two EhuR operator sequences resemble each other closely and fit to the EutR-type consensus sequence defined by Suvorova and Rodionov (2016) from the analysis of 15 genome sequences of *Rhizobiales* (Suvorova and Rodionov, 2016) (Fig. 5B). Although not specifically mentioned in their report on ectoine utilization by *H. elongata* (Schwibbert *et al.*, 2011), this bacterium actually possesses an EnuR-related protein (34% sequence identity to EnuR from *R. pomeroyi*) whose structural gene is positioned next to the catabolic gene cluster. These authors mapped the transcriptional start site of the ectoine catabolic gene cluster (Schwibbert *et al.*, 2011) and we found through DNA-sequence inspection a putative EnuR binding site in its vicinity (Fig. 5B).

To provide further evidence that the intergenic region between the *enuR* and *uehA* genes of *R. pomeroyi* contains the operator(s) for EnuR, we carried out DNA-band-shift assays with affinity-chromatography-purified EnuR. The heterologous produced protein contained PLP, as judged by its intense yellow colour (Schulz *et al.*, 2017).

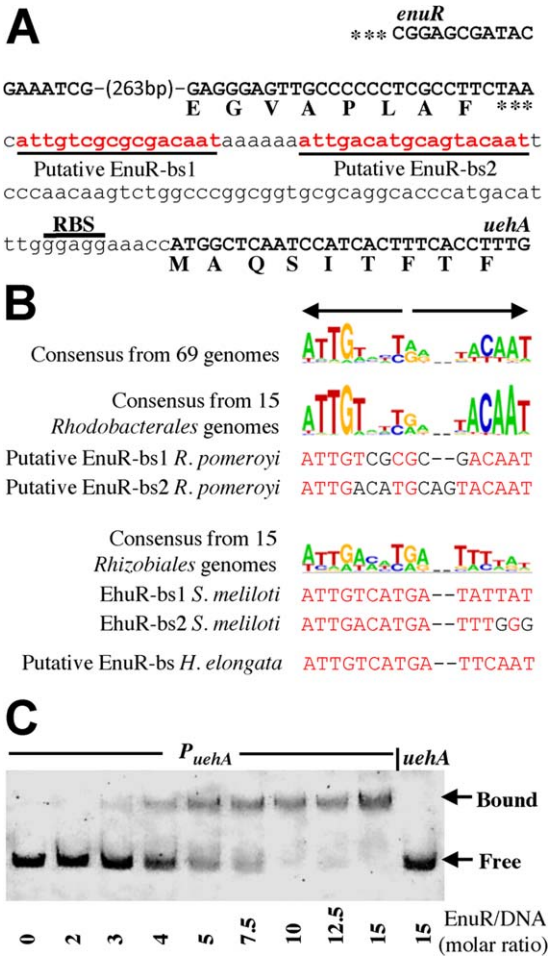

Fig. 5. *In silico* analysis of putative EnuR binding sites and interactions of EnuR with DNA. (A) DNA sequence of the 3'-end of the *enuR* gene, the *enuR-uehA* intergenic region and the beginning of the *uehA* gene. The end of the shown *uehA* DNA sequence corresponds to the *uehA/lacZ* junction present in the transcriptional *lacZ* reporter gene fusion plasmids pBAS20, pBAS21 and pBAS23. The predicted two EnuR binding sites and the ribosome-binding site (RBS) of the *uehA* gene are indicated. (B) DNA sequence logos of *in silico* predicted EutR-type binding-sites (Suvorova and Rodionov, 2016) and those for EnuR (this study), the EhuR protein from *S. meliloti* as determined by DNase I footprinting analysis (Yu *et al.*, 2017), and as identified by us from previously reported promoter-mapping experiments of the ectoine catabolic gene cluster of *H. elongata* (Schwibbert *et al.*, 2011). (C) Electrophoretic DNA-band-shift assays of a 251-bp DNA fragment with affinity-purified EnuR protein. 0.5 pmol of the fluorescently labelled DNA fragment carrying the *enuR-uehA* intragenic region was incubated with increasing concentrations of affinity-purified EnuR protein; subsequently, the DNA fragment incubated in the absence of EnuR and the formed DNA:EnuR complexes were electrophoretically separated and visualized by imaging. A 278-bp DNA fragment derived from the coding region of *uehA* that should not contain any EnuR binding sites was used as a control.

To this end, we incubated increasing EnuR concentrations with 0.5 pmol of a 251 bp DNA fragment that carries part of the 3'-end of the *enuR* gene, the 97 bp *enuR-uehA* intergenic region, and 14 codons of *uehA* and visualized the formed DNA:EnuR complexes after non-denaturing gel electrophoresis. DNA:EnuR complexes began to form with a concentration of EnuR as little as 75 nM (Fig. 5C). These interactions were specific, because there was no DNA mobility shift of a 0.5 pmol DNA fragment (278 bp) derived from the *uehA* coding region (Fig. 5C).

Influence of hydroxyectoine and ectoine importer and catabolic genes on uehA-lacZ reporter gene expression

The above reported data show that internal inducers, *N*-(α)-ADABA and DABA, for the EnuR regulatory protein are generated through the catabolism of ectoines. To study this issue further, we used a set of strains in which the hydroxyectoine/ectoine UehABC transporter system (Lecher *et al.*, 2009) or various catabolic genes (Schulz *et al.*, 2017) were defective and employed the transcriptional activity of the *uehA-lacZ* reporter fusion present on pBAS20 as a read-out for these experiments. First, we tested a strain (ASR6) in which the entire hydroxyectoine/ectoine importer and catabolic cluster was deleted and found that induction of *uehA-lacZ* expression in response to the presence of either ectoine or hydroxyectoine was no longer possible (Fig. 6A).

In a strain (ASR12) in which the *uehABC* transporter genes are intact but those for the catabolism of ectoines are deleted, reasonably good induction of gene expression by ectoine (8.6-fold in the mutant versus 14-fold in the wild-type) can be observed, whereas that afforded by hydroxyectoine is greatly diminished (8.3-fold in the mutant versus 64-fold in the wild-type) (Fig. 6B). A similar pattern of induction of *uehA-lacZ* expression was observed when only the *eutABC* genes were deleted; these genes are required for the growth of *R. pomeroyi* on 5-hydroxyectoine but not on ectoine (Supporting Information Fig. S2). The loss of the EutABC enzymes had a major impact on the induction of *uehA-lacZ* expression in response to the availability of hydroxyectoine whereas that afforded by the presence of ectoine was only moderately affected (Fig. 6C). When *eutD* was deleted (strain ASR8), a mutation that abolishes the use of both ectoines as nutrients (Supporting Information Fig. S2), induction of *uehA-lacZ* expression in response to ectoine occurred at a substantially reduced level (7.7-fold in the mutant versus 15.2-fold in the wild-type) but the strong induction of transcription afforded by hydroxyectoine (60-fold) was unaffected (Fig. 6D).

These findings are both surprising and informative in several aspects when one considers that the EutD enzyme is required to generate the internal inducers *N*-(α)-ADABA and DABA for EnuR (Figs. 1 and 4A and B). First, our observation that induction of gene expression (at least to some extent) in response to ectoine can still occur in an *eutD* mutant (Fig. 6D) and in a mutant in which all catabolic genes are deleted but in which those for the UehABC transporter were intact (Fig. 6B) indicates that genetic and

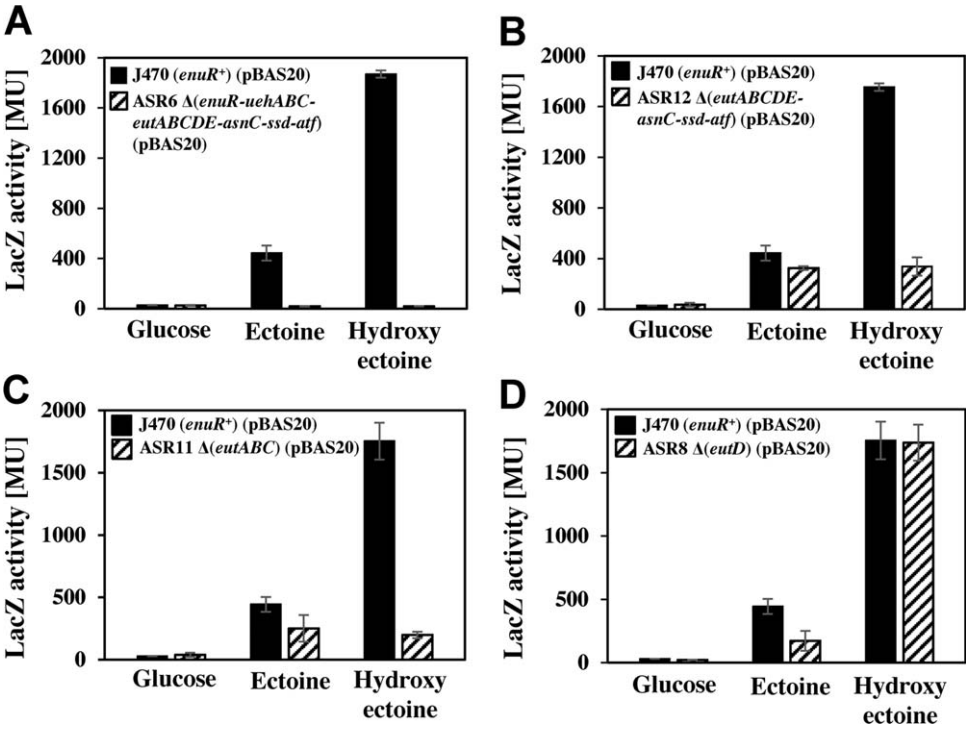

Fig. 6. Substrate induction of *uehA-lacZ* expression in response to hydroxyectoine and ectoine availability and the influence of various chromosomal mutations. The pattern of substrate induction of the *uehA-lacZ* reporter fusion present on plasmid pBAS20 by either hydroxyectoine or ectoine was assessed in strains carrying either (A) a complete deletion of the hydroxyectoine/ectoine importer and catabolic genes (strain ASR6), (B) the *ehuABC*$^{+}$*-eutABCDE-asnC-ssd-atf strain* ASR12, (C) the Δ(*eutABC*) mutant strain ASR11 and (D) the Δ(*eutD*) mutant strain ASR8.

physiological input into the regulatory system occurs beyond the generation of the internal inducers *N*-(α)-ADABA and DABA through ectoine catabolism. Second, our finding that induction of gene expression in response to hydroxyectoine occurs unabated in an *eutD* mutant, while that by ectoine is negatively affected (Fig. 6D), suggests that an internal inducer other than *N*-(α)-ADABA and DABA is generated through the break-down of hydroxyectoine. Indeed, loss of the *eutABC* gene not only abolished use of hydroxyectoine as a nutrient (Supporting Information Fig. S2) but simultaneously severely affected transcription of the *uehA-lacZ* reporter fusion as well (Fig. 6C). All these complex regulatory effects depend on the import of ectoine or hydroxyectoine (Fig. 6A and B).

The feast-and-famine regulator AsnC has a major influence on the expression of the importer and catabolic gene cluster

In most (494 out of the 539) of the importer and catabolic gene clusters of previously predicted microbial hydroxyectoine/ectoine consumers, a gene for the *feast-and-famine* type regulator AsnC is present (Schulz *et al.*, 2017) (Supporting Information Fig. S3). These types of proteins can form octamers wrapping their target DNA around them into a high-ordered tertiary nucleosome-like complex, and their DNA-binding properties are dictated through interactions with low-molecular-mass effector molecules, often amino acids (Yokoyama *et al.*, 2006; Shrivastava and Ramachandran, 2007; Kumarevel *et al.*, 2008; Kamensek *et al.*, 2015; Dey *et al.*, 2016). We have previously demonstrated that the deletion of the last two genes (*ssd-atf*) in the hydroxyectoine/ectoine catabolic operon (Fig. 1) is functionally substituted by other genes from *R. pomeroyi*, and has in essence no effect on the use of ectoines as nutrients (Fig. 7C). In contrast, the simultaneous deletion of the *asnC-ssd-atf* genes abolishes consumption of ectoine when it was provided as joint carbon and nitrogen source (Schulz *et al.*, 2017). We now found that AsnC was required for use of ectoine as sole carbon source but was dispensable for its use as sole nitrogen source (Fig. 7C). Collectively, these growth data imply an important regulatory role for the AsnC protein encoded in the *R. pomeroyi* 5-hydroxyectoine/ectoine importer and catabolic gene cluster.

Consistent with the previously reported growth data (Schulz *et al.*, 2017), we found that ectoine- or hydroxyectoine-mediated induction of *uehA-lacZ* expression in an *asnC*$^+$-Δ(*ssd-atf*) strain (ASR14) still reached about 66% (for ectoine) and 75% (for hydroxyectoine), respectively, of the level observed in a wild-type strain (Fig. 7A). In striking contrast, induction of *uehA-lacZ* expression in the Δ(*asnC-ssd-atf*) strain ASR10 by either ectoine or hydroxyectoine was reduced substantially further, reaching approximately 50% of the wild-type level with ectoine, and only 16% with hydroxyectoine (Fig. 7B). This strong positive influence of the AsnC regulatory protein on transcription of the importer and catabolic gene cluster is reflected by the inability of the *asnC* mutant to exploit ectoine as sole carbon source (Fig. 7C).

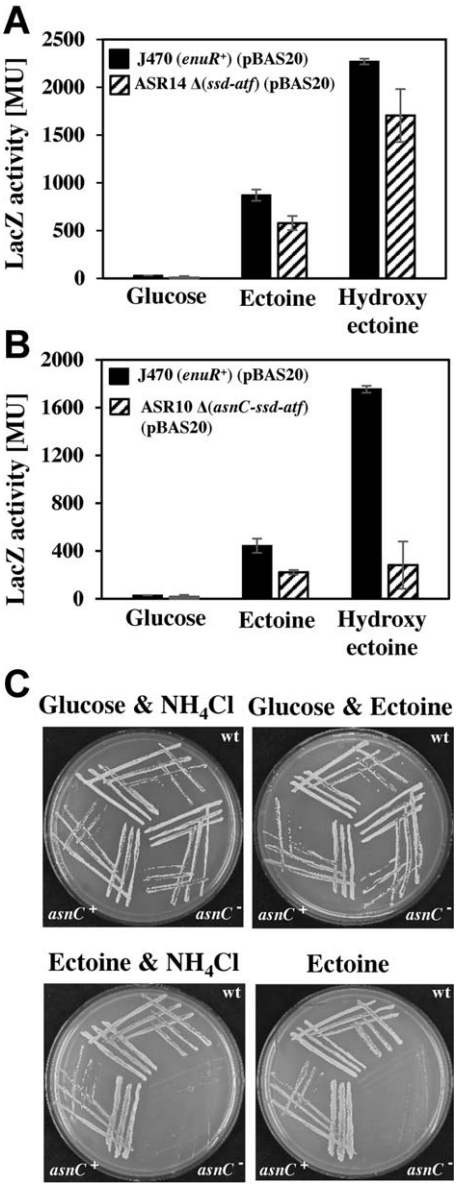

Fig. 7. Role of the AsnC regulator for substrate-mediated induction of *uehA-lacZ* expression and the use of ectoine as a nutrient. The pattern of substrate induction of the *uehA-lacZ* reporter fusion present on plasmid pBAS20 by hydroxyectoine or ectoine was assessed in strains carrying either a chromosomal (A) Δ(*ssd-atf*) mutation (strain ASR14) or (B) a strain (ASR10) in which the *asnC* gene was additionally deleted [Δ(*asnC-ssd-atf*)]. (C) Use of ectoine as nutrient by the *R. pomeroyi* wild-type strain J470 (WT), strain ASR14 [*asnC*$^+$-Δ(*ssd-atf*)] (*asnC*$^+$) and strain ASR10 [Δ(*asnC-ssd-atf*)] (*asnC*$^-$).

Regulatory input of the nitrogen-sensing NtrYX two-component system into the genetic control of import and catabolism of ectoines

The tetrahydropyrimidines hydroxyectoine and ectoine are nitrogen-containing compounds (Fig. 1) and can be used by *R. pomeroyi* as sole sources of this essential element (Schulz *et al.*, 2017) (Fig. 7C). We, therefore, wondered if the expression of the hydroxyectoine and ectoine import and catabolic gene cluster would be under the genetic control of sensory systems monitoring the availability of nitrogen-containing compounds in the environment. To search for such regulatory systems, we carried out a transposon mutagenesis of *R. pomeroyi* DSS-3 using the EZ-Tn*5*^{TM} transposition system (Epicenter, Madison, USA) (Goryshin and Reznikoff, 1998) and searched the resulting EZ-Tn*5*^{TM} transposon insertion collection for mutant strains unable to use ectoine as a nutrient on agar plates where ectoine was the sole available carbon and nitrogen source. From approximately 21 000 colonies that were inspected, we identified three colonies with a defect in ectoine utilization. Molecular analysis showed that these three strains contained the EZ-Tn*5* transposon at the same position in codon 189 of the *ntrY* gene (Fig. 8A). The recovered *ntrY*::EZ-Tn*5* insertion mutants thus either are siblings or result from a hot-spot of Tn*5* integration into the *R. pomeroyi* chromosome. The *ntrY* gene encodes a 762-amino-acid-comprising integral membrane protein that serves as the sensor-kinase for a two-component regulatory system (NtrYX) implicated, among several other cellular processes, in the catabolism of nitrogen-containing compounds (Fernandez *et al.*, 2017). On further molecular analysis, we found that the *ntrY*::EZ-Tn*5* insertion mutant strains obtained in the above described genetic screen contained multiple EZ-Tn*5* copies, making it impossible to employ these strains for clean genetic and physiological studies.

Taking the data from the transposon mutagenesis as a lead, we constructed a genetically precisely defined chromosomal deletion/insertion mutation (strain ASR9) destroying the NtrYX system entirely [Δ(*ntrYX::Gm*^{R})1] through recombinant DNA techniques. We found that this engineered mutation abolished the use of ectoine as sole carbon source but still permitted the use of ectoine as sole nitrogen source (Fig. 8B). We introduced the *uehA-lacZ* reporter fusion plasmid pBAS20 into the [Δ(*ntrYX::Gm*^{R})1] mutant strain ASR9 and studied the influence of this gene disruption mutation on the transcriptional activity of the *uehA* promoter in response to the availability of ectoines. The transcriptional profile of strain ASR9 (pBAS20) differed significantly from that of the wild-type strain. Loss of the NtrYX two-component regulatory system allowed only a reduced level of induction of gene expression, with the inducing effects of hydroxyectoine being the most strongly affected (Fig. 8C). We note, that the remaining level of transcription of these genes in the absence of the NtrYX system (Fig. 8C) was insufficient to allow *R. pomeroyi* the use of ectoines as the sole carbon source (Fig. 8B).

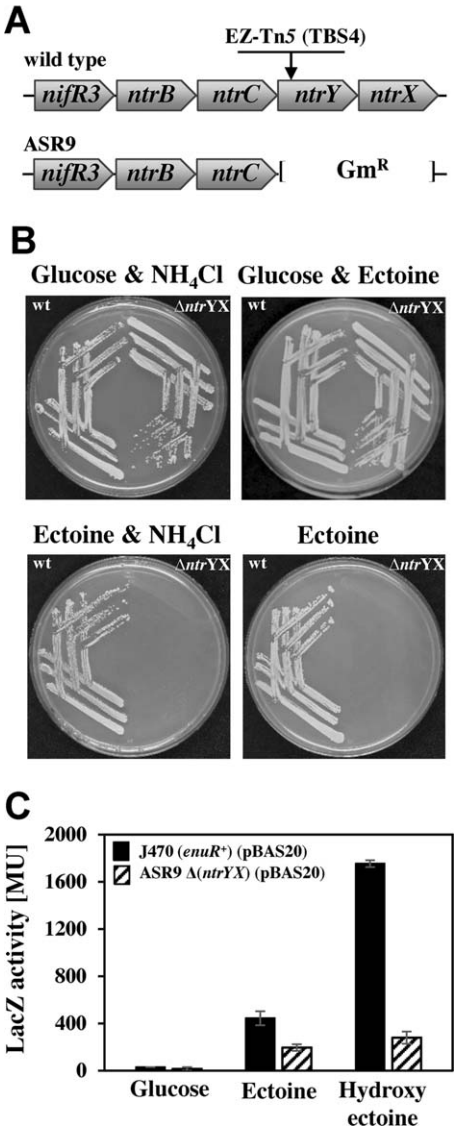

Fig. 8. Role of the NtrXY two-component regulatory system for growth of *R. pomeroyi* on ectoine and substrate-mediated induction of *uehA-lacZ* expression. (A) Genetic organization of the *nifR3-ntrB-ntrC-ntrY-ntrX* region of the *R. pomeroyi* genome (Moran *et al.*, 2004), the position of the *ntrY*::EZ-Tn*5* insertion in strains unable to use ectoine as nutrients, and the *in vitro* constructed Δ(*ntrYX::Gm*^{R}) deletion mutation in strain ASR9. (B) Growth of the *R. pomeroyi* wild-type strain J470 and its Δ(*ntrYX::Gm*^{R}) mutant derivative (strain ASR9) on basal minimal medium agar plates containing 28 mM ectoine when used either as sole carbon or nitrogen source. When the use of ectoine as joint carbon and nitrogen sources was tested, the plates contained 56 mM ectoine. (C) The pattern of substrate induction of the *uehA-lacZ* reporter fusion present on plasmid pBAS20 by either hydroxyectoine or ectoine was assessed either in the wild-type strain J470 or in its Δ(*ntrYX::Gm*^{R}) mutant derivative strain ASR9.

R. pomeroyi can exploit the osmolytes glycine betaine and choline as nutrients (Lidbury *et al.*, 2015). We found that the disruption of the NtrYX system has no influence on the use of these nitrogen-containing compounds when they were tested either as sole carbon or nitrogen sources (Supporting Information Fig. S4).

Discussion

The co-occurrence of microbial hydroxyectoine/ectoine producers and consumers in the same habitat (Schwibbert *et al.*, 2011; Widderich *et al.*, 2014; 2016b; Schulz *et al.*, 2017) drives ecophysiological relevant networks of synthesis, release and catabolism of compounds that are produced in abundance by many osmotically stressed bacterial cells (Pastor *et al.*, 2010; Kunte *et al.*, 2014; Widderich *et al.*, 2014). For the exploitation of externally provided ectoines as nutrients, it is essential that the consumers can sensitively detect the presence of these compounds in their surroundings (Welsh, 2000; Mosier *et al.*, 2013; Warren, 2014; 2016), so that they can trigger enhanced expression of those genes whose products mediate import and catabolism of ectoines (Jebbar *et al.*, 2005; Lecher *et al.*, 2009; Schwibbert *et al.*, 2011; Schulz *et al.*, 2017; Yu *et al.*, 2017).

The data presented here address this issue in the metabolically versatile marine bacterium *R. pomeroyi* DSS-3 (Luo and Moran, 2014) and identify three regulators that contribute to the transcriptional control of hydroxyectoine/ectoine import and catabolism: the GabR/MocR-type repressor EnuR, the *feast-and-famine*-type regulator AsnC, and the two-component regulatory system NtrYX (Fig. 9A). In Supporting Information Fig. S3, we have projected onto a EutD-based phylogenetic tree (Schulz *et al.*, 2017) the distribution of these three regulators among 539 proteobacterial potential consumers of ectoines. There is considerable overlap between the distribution of *enuR*, *asnC* and *ntrYX* genes in the genomes of ectoine-consuming microorganisms (Fig. 9B), and notably, a very substantial group (about 45%) of them possesses all three regulators (EnuR, AsnC, NtrYX). While the *enuR* and *asnC* genes are widely distributed among all branches of ectoine-degrading *Proteobacteria*, the *ntrYX* genes are notably restricted to all ectoine-consuming members of the *Alphaproteobacteria* (Supporting Information Fig. S3). However, it appears from additional database searches that the presence of the NtrYX system is a common trait in *Alphaproteobacteria* and this includes also species unable to catabolize ectoines.

Our genetic and molecular data identify EnuR as a key regulator for the use of ectoines as nutrients. Not only is the *enuR* gene commonly (85%) associated with hydroxyectoine/ectoine catabolic gene clusters (Fig. 9 and Supporting Information Fig. S3), its regulatory function

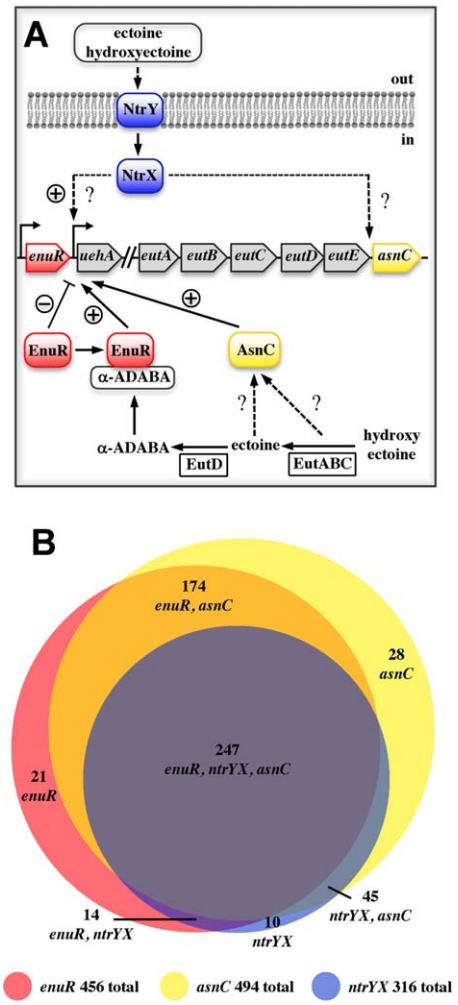

Fig. 9. Distribution of *enuR*, *asnC* and *ntrXY* genes in predicted hydroxyectoine/ectoine consumers and a working model for genetic regulation of hydroxyectoine/ectoine uptake and catabolic genes. (A) Working model for the genetic control of hydroxyectoine/ectoine uptake and catabolic genes in *R. pomeroyi*. (B) A previously assembled dataset of 539 predicted hydroxyectoine/ectoine consumers, all of which belong to the *Proteobacteria* (Schulz *et al.*, 2017) (Supporting Information Fig. S3), were inspected for the presence of *enuR*-type genes in the vicinity of the hydroxyectoine/ectoine uptake and catabolic gene clusters, for the presence of *asnC*-type genes within these gene clusters, and for the presence of *ntrXY*-type genes elsewhere in the genome sequences of this group of microorganisms.

also depends on an effector molecule [*N*-(α)-ADABA] that is exclusively generated during ectoine catabolism (Fig. 1). EnuR is a member of the widely distributed family of MocR/GabR-type transcriptional regulators (Bramucci *et al.*, 2011; Suvorova and Rodionov, 2016), and like other members of this family contains a covalently attached PLP co-factor in its carboxy-terminal aminotransferase domain (Schulz *et al.*, 2017). We found that the bound PLP is indispensable for the recognition of the *N*-(α)-ADABA effector molecule (Fig. 4A and Supporting Information Fig. S1) and

for the ability of this internal inducer to relieve EnuR-mediated repression of transcription (Fig 3B). Not surprisingly, the residue to which the PLP co-factor is attached (corresponding to K302 in the *R. pomeroyi* EnuR protein) is strictly conserved in an alignment of 456 EnuR-type proteins. Consistent with data reported for the EnuR orthologue of *S. meliloti* (Yu *et al*., 2017), DABA serves also as an inducer for the EnuR protein of *R. pomeroyi*. However, the binding affinities of EnuR for DABA are substantially reduced (about 230-fold) in comparison with *N*-(α)-ADABA (Fig. 4A and B). *N*-(α)-ADABA can thus be regarded as the primary internal inducer for hydroxyectoine/ectoine uptake and catabolism.

Apart from the high affinity of EnuR for *N*-(α)-ADABA, this compound has the additional advantage of being a specific ectoine-derived metabolite (Fig. 1), whereas DABA occurs also as an intermediate in other metabolic and biosynthetic processes in microorganisms (Ikai and Yamamoto, 1997; Du *et al*., 2013; Fidalgo *et al*., 2016), including the biosynthesis of ectoine (Ono *et al*., 1999) (Fig. 1). In the K302/H substitution variant of EnuR (EnuR*), the covalent attachment of the PLP molecule is no longer possible (Schulz *et al*., 2017). As shown here, this turns the EnuR* mutant protein into a negative dominant super-repressor unable to respond to externally provided ectoines (Fig 3B). EnuR* apparently binds to DNA *in vivo* so tightly that the use of ectoines as nutrients is abolished (Fig. 3C and D). The pairing of the EnuR repressor with the attached PLP co-factor and ectoine-derived metabolites [*N*-(α)-ADABA and DABA] establishes a sensitive intracellular trigger to relieve EnuR-mediated repression. In contrast to *N*-(α)-ADABA, its isomer *N*-(γ)-ADABA is not bound by EnuR (Supporting Information Fig. S1C). This is a significant finding as *N*-(γ)-ADABA is an intermediate in ectoine biosynthesis (Fig. 1) (Ono *et al*., 1999; Widderich *et al*., 2016a). This discovery is particularly relevant for the substantial group of microorganisms that are capable of both ectoine synthesis and catabolism (Schwibbert *et al*., 2011; Schulz *et al*., 2017).

Recent detailed biochemical and structural studies with the PLP-containing and GABA-responsive GabR regulatory protein from *B. subtilis* (Edayathumangalam *et al*., 2013) have significantly enhanced our understanding of the roles played by the PLP molecule and the respective system-specific inducer of MocR/GabR-type regulators (Okuda *et al*., 2015a,b; Park *et al*., 2017; Wu *et al*., 2017). The side chain of K312 of GabR forms an internal aldimine with PLP and further chemical reactions of the system-specific inducer GABA with the bound PLP molecule leads to a formation of a PLP-GABA adduct (the external aldimine) with the concomitant release of PLP from the side chain of K312. This sequence of events triggers a structural transition in the entire regulatory protein affording a change in GabR-mediated regulation of transcription (Park *et al*., 2017; Wu *et al*., 2017). The GabR protein is a head-to-tail dimer (Edayathumangalam *et al*., 2013) and the structural transitions caused by the formation of the external aldimine have recently been captured in the crystal structure of the GabR carboxy-terminal effector binding/oligomerization domain where one of the monomers contains the internal aldimine and the other subunit harbours the external aldimine (Park *et al*., 2017).

Based on the extensive studies of PLP-mediated inducer binding by GabR, and related studies with the D-alanyl-D-alanine-responsive MocR/GabR-type regulator DdlR from *Bacillus brevis* (Takenaka *et al*., 2015), we propose here that the basal form of EnuR containing the internal aldimine between K302 and PLP changes to an external aldimine form when *N*-(α)-ADABA is produced through ectoine catabolism and binds to the K302-attached PLP co-factor. The ensuing conformational change of EnuR will then trigger changes in the DNA-binding properties of this regulatory protein, thereby causing an increase in transcription of the hydroxyectoine/ectoine uptake and catabolic gene cluster (Fig. 4C). A similar reaction scheme can readily be envisioned for the secondary internal inducer of EnuR, DABA, as well (Fig. 1).

Without the proper functioning of the *eutD*-encoded ectoine hydrolase, the internal inducers *N*-(α)-ADABA and DABA cannot be formed through the catabolism of ectoine. The catabolism of hydroxyectoine is envisioned to proceed through the transient formation of ectoine (Fig. 1). It follows from the proposal of this catabolic route (Schulz *et al*., 2017) that an external supply of hydroxyectoine should no longer lead to the expression of the *ueh-lacZ* reporter gene fusion if its inducing effect is also dependent on EnuR and the EutD-generated formation of *N*-(α)-ADABA and DABA. However, in contrast to expectations, there is strong induction of *ueh-lacZ* transcription in response to hydroxyectoine in a *eutD* mutant strain (Fig. 6D). Interestingly, induction by hydroxyectoine is largely abolished in a strain lacking the *eutABC* genes (Fig. 6C), which encode the enzymes required for conversion of hydroxyectoine to ectoine and growth of *R. pomeroyi* on hydroxyectoine as the sole carbon, nitrogen and energy source (Supporting Information Fig. S2). In agreement with the proposed overall route for the catabolism of ectoines (Schulz *et al*., 2017) (Fig. 1), loss of the EutABC enzymes still permits utilization of ectoine as a nutrient (Supporting Information Fig. S2). Ectoine also still triggers the induction of *uehA-lacZ* reporter gene expression in the corresponding mutant strain, albeit at a somewhat reduced level (Fig. 6C), a phenomenon that might be caused by a partially polar effect of the *eutABC::Gm*^{R} deletion/insertion mutation on the transcription of the down-stream located ectoine catabolic genes (Fig. 1).

The internal inducer generated from the catabolism of hydroxyectoine through the enzymatic activities of the

EutABC proteins is substantially more potent than the EnuR ligands *N*-(α)-ADABA and DABA (Figs. 2B, C, and 6A). Notably, induction of *ueh-lacZ* reporter gene expression by hydroxyectoine is greatly reduced in a Δ(*asnC-ssd-atf*) mutant strain (Fig. 7B), while an external supply of this compound triggers a very strong induction of the reporter fusion in a *asnC*$^{+}$-Δ(*ssd-atf*) strain (Fig. 7A). Combined with our finding that induction of *ueh-lacZ* expression is dependent on the activity of the EutABC enzymes (Fig. 6C), we are left with the conclusion that the metabolism of hydroxyectoine generates a compound(s) that might serve as an effector molecule for AsnC (Fig. 9B). Ligand binding assays with AsnC are required to verify or refute this genetically derived hypothesis, experiments not easy to conduct since none of the predicted intermediates in hydroxyectoine catabolism to ectoine (Fig. 1) are commercially available.

AsnC exerts a clear activating influence on the expression of the *R. pomeroyi* hydroxyectoine/ectoine-importer and catabolic gene cluster. AsnC/Lrp-type proteins wrap DNA into nucleosome-like structures and frequently respond in their DNA-binding properties to low-molecular-mass effector molecules that are generated through metabolism (e.g., amino acids) (Shrivastava and Ramachandran, 2007; Kumarevel *et al*., 2008; Dey *et al*., 2016). In many cases, these proteins respond to *feast-and-famine* situations and thereby permit the efficient exploitation of sudden burst in the supply of a particular nutrient in fluctuating environmental settings (Yokoyama *et al*., 2006). Interestingly, Landa *et al*. (2017) recently reported a major remodelling of the *R. pomeroyi* DSS 3 transcriptome, depending whether this bacterium was co-cultivated with the dinoflagellate *Alexandrium tamarense* or the diatom *Thalassosira pseudonana*. Depending on which phytoplankton species dominated the co-culture, the ectoine uptake and catabolic genes were differentially expressed; the presence of the diatom triggered enhanced transcription of these genes, hinting to a *T. pseudonana*-derived source of ectoine for use as a nutrient by *R. pomeroyi* DSS 3 (Landa *et al*., 2017).

Initial DNA-binding studies with an AsnC orthologue (referred to as DeoX) of *H. elongata* revealed interaction(s) with the presumed regulatory region of the ectoine catabolic gene cluster (Schwibbert *et al*., 2011). Relevant to a discussion about the role of hydroxyectoine/ectoine catabolism associated AsnC-type proteins is the fact that AsnC/Lrp-type proteins can also work in concert with other types of regulators (Kamensek *et al*., 2015), a facet in gene regulation that probably becomes highly relevant for the large group of microbial ectoine consumers that possesses both EnuR and AsnC (about 85% in our data-set) (Fig. 9B). Nevertheless, 73 of 494 AsnC-containing representatives lack EnuR (Fig. 9B and Supporting Information Fig. S3), indicating that the regulatory roles played by EnuR and AsnC are not necessarily mutually interdependent.

The data presented here also identify a novel player (NtrYX) in the genetic control of hydroxyectoine/ectoine-catabolism by *R. pomeroyi* where this two-component regulatory system serves as a positive regulator of gene expression. Two-component regulatory systems such as NtrYX serve as major signalling devices of microorganisms through which information about changes in the environment are detected, processed and then transmitted to the transcriptional apparatus of the cell (Zschiedrich *et al*., 2016). The NtrYX system (Fig. 9A) has been implicated in a number of biologically rather varied cellular processes in *Alphaproteobacteria* [for a recent description of the NtrYX system and additional references see (Fernandez *et al*., 2017)], notably also in the metabolism and assimilation of nitrogen-containing compounds (Pawlowski *et al*., 1991; Carrica Mdel *et al*., 2012; Cheng *et al*., 2014; Bonato *et al*., 2016; Calatrava-Morales *et al*., 2017). Our data now subscribe a function to the NtrYX system of *R. pomeroyi* with respect to the catabolism of ectoine when it is used as sole carbon source while still allowing its use as sole nitrogen source (Fig. 8B). These differences in the use of ectoine as a nutrient by the *R. pomeroyi ntrYX* mutant might be caused by the facts that (i) more carbon than nitrogen units are required for growth and (ii) that a *ntrYX* deletion mutant is not completely deficient in the induction of the hydroxyectoine/ectoine import and catabolic gene cluster in response to both ectoines (Fig. 8C). We do not know yet whether the influence of the NtrYX system on the utilization of ectoines by *R. pomeroyi* are mediated through direct interactions of the unusual NtrC-type NtrX response regulator (Zschiedrich *et al*., 2016; Fernandez *et al*., 2017) with the regulatory region present in front of the *R. pomeroyi* hydroxyectoine/ectoine-uptake and catabolic gene cluster (Fig. 9A), or whether they are mediated indirectly through a so far undisclosed regulatory circuit (e.g., by controlling the expression of *asnC* or AsnC activity).

With the exception of the deletion that removes the hydroxyectoine/ectoine-importer and metabolic genes altogether, none of the single *enuR*, *asnC* and *ntrYX* gene disruption mutations abolishes hydroxyectoine/ectoine-responsive *uehA-lacZ* reporter gene expression entirely. These hints to multiple levels of input of ectoines into the signal perception and transduction process and the data reported here paint a rather complex picture of the finely tuned genetic control of hydroxyectoine/ectoine import and catabolism. In Fig. 9A, we present a regulatory scheme that is consistent with our experimental data and formulates experimentally testable hypothesis. The physical organization and the precise gene content of hydroxyectoine/ectoine uptake and catabolic gene clusters vary (Schwibbert *et al*., 2011; Schulz *et al*., 2017), indicating that alternatives to our proposal (Fig. 1) for the catabolism

of hydroxyectoine to ectoine likely exist in microorganisms (Schulz *et al*., 2017). The data and consideration we present here indicate that their transcriptional regulation probably entails variations of a common theme as well.

Experimental procedures

Chemicals

Ectoine was kindly provided by the bitop AG (Witten, Germany) and hydroxyectoine was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anhydrotetracycline hydrochloride (AHT), desthiobiotin and Strep-Tactin Superflow chromatography material were obtained from IBA GmbH (Göttingen, Germany). The β-galactosidase substrate, o-nitrophenyl-β-*D*-galactopyranosid (ONPG), and the antibiotics gentamycin, spectinomycin, rifampicin and kanamycin were obtained from Serva (Heidelberg, Germany). Ampicillin was purchased from Carl Roth GmbH (Karlsruhe, Germany). The α- and γ-isomers of *N*-acetyl-L-2,4-diaminobutyric acid (ADABA) were prepared through alkaline hydrolysis of ectoine (Kunte *et al*., 1993) and their separation was accomplished through repeated chromatography on a silica gel column (Merck silica gel 60). The identity and purity of these compounds was established by thin-layer chromatography and nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectroscopy as detailed previously (Widderich *et al*., 2016a).

Media and growth conditions

Ruegeria pomeroyi strains (Supporting Information Table S1) were maintained on half-strength YTSS agar. When required, gentamycin was added to the agar plates. For all growth experiments, strains of *R. pomeroyi* were cultivated in defined basal minimal medium (Baumann *et al*., 1971). This medium had the following composition: 50 mM MOPS (pH 7.5), 200 mM NaCl, 10 mM KCl, 330 μM K_2HPO_4, 10 mM $CaCl_2$, 50 mM $MgSO_4$ and 100 μM $FeSO_4$. To this basal medium we added a stock solution (200-fold concentrated) of vitamin mixture A and vitamin mixture B. The final concentrations of the vitamins were for mixture A 39 μg l^{-1} biotin, 78 μg l^{-1} nicotinic acid, 78 μg l^{-1} lipoic acid and 78 μg l^{-1} folic acid and for mixture B 78 μg l^{-1} pantothenic acid, 78 μg l^{-1} pyridoxine, 78 μg l^{-1} thiamine, 78 μg l^{-1} 4-aminobenzoic acid and 1.6 μg l^{-1} cobalamin. We also added 0.1 mM methionine, 0.1 mM serine and 0.1 mM glutamate to the growth medium since, according to our experience, these amino acids significantly improved the growth of *R. pomeroyi* strains. Glucose (28 mM) and NH_4Cl (200 mM) were routinely used as the carbon and nitrogen sources for *R. pomeroyi* DSS-3 in liquid medium. When either ectoine or hydroxyectoine were used as combined carbon and nitrogen sources in liquid cultures, NH_4Cl and glucose were left out of the medium and ectoines were provided at a final concentration of 28 mM. When *R. pomeroyi* cells were plated on basal medium agar plates, glucose (28 mM) and NH_4Cl (56 mM) were used as carbon and nitrogen sources. When ectoine was tested as a carbon or nitrogen source on such agar plates, ectoine was used at a concentration of 28 mM as a substitute for glucose or NH_4Cl, and when it was simultaneously used as carbon and nitrogen source, we added 56 mM to growth media. When the use of choline or glycine betaine was tested as sole carbon or nitrogen sources, they were added to basal medium agar plates at a final concentration of 28 mM (when tested as sole carbon sources), at 56 mM (when tested as sole nitrogen sources), and at 84 mM (when tested as joint carbon and nitrogen sources). When applicable, the antibiotics gentamycin, spectinomycin, rifampicin, or kanamycin were added to liquid and solid media at concentrations of 20, 150, 20 and 80 μg ml^{-1} respectively. Liquid cultures of *R. pomeroyi* and agar plates streaked with this microorganism were grown at 30°C.

Plasmids containing the *enuR*$^{+}$ or *enuR** genes were routinely maintained in the *Escherichia coli* strain DH5α (Invitrogen, Karlsruhe, Germany) on LB agar plates containing ampicillin (100 μg ml^{-1}). Minimal Medium A (MMA) (Miller, 1972) containing 0.5% (w/v) glucose as the carbon source, 0.5% (w/v) casamino acids (0.5%), 1 mM $MgSO_4$ and 3 mM thiamine was used for cultivation of the *E. coli* B strain BL21 carrying plasmids pBAS3 (*enuR*$^{+}$) or pBAS17 (*enuR**) (Supporting Information Table S2) for the overproduction of the EnuR protein and its EnuR* mutant derivative (a K302/H amino acid substitution) (Schulz *et al*., 2017).

Bacterial strains and plasmids

The *E. coli* strain DH5α was used for routine cloning purposes and the *E. coli* B strain BL21 (DE3) (Stratagene, La Jolla, CA) was employed for the overexpression of the *R. pomeroyi enuR* gene and its mutant *enuR** derivative. 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 of this isolate (strain J470) (Todd *et al*., 2012) was kindly provided by J. Todd and A. Johnston (University of East Anglia, United Kingdom). *E. coli* DH5α strains carrying the helper plasmid pRK2013 [Kan^{R}] (Figurski and Helinski, 1979) for conjugation experiments between *E. coli* and *R. pomeroyi*, and the *lacZ* reporter fusion plasmid pBIO1878 [Spc^{R}] (Todd *et al*., 2012) were also provided by these colleagues.

Recombinant DNA techniques and construction of plasmids

Chromosomal DNA of *R. pomeroyi* DSS-3 was isolated as described (Schulz *et al*., 2017). The High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany) was used to isolate plasmid DNA from *E. coli* strains. Restriction endonucleases and DNA ligase were obtained from ThermoScientific (St. Leon-Rot, Germany) and used as suggested by the manufacturer. Chemically competent cells of *E. coli* were prepared and transformed as reported previously (Sambrook *et al*., 1989). To construct a *uehA-lacZ* reporter gene fusion that also carried the intact *enuR* gene, a 2.6-kb chromosomal DNA fragment from strain DSS-3 was amplified by PCR using Phusion DNA polymerase (Life Technologies, Darmstadt, Germany) and the custom-synthesized DNA primers LacZenuR_up_for and LacZuehA_PstI_rev (Supporting Information Table S3). After purification of this DNA fragment using the QIA Quick PCR Purification Kit (Qiagen, Hilden, Germany), it was digested with EcoR1 and Pst1 and ligated into the linearized broad-host-range *lacZ*-fusion vector pBIO1878 (Todd

et al., 2012) that had also been cut with these enzymes. This yielded the *enuR*$^{+}$*-uehA-lacZ* reporter plasmid pBAS20; it encompasses the complete *enuR* gene, the 97-bp *enuR-uehA* intergenic region and 28 bp of the *uehA* coding sequence. A variant of pBAS20 carrying a mutation [AAA/CAT] that leads to the substitution of K302 of EnuR by a H residue (EnuR*) was constructed through site-directed mutagenesis using the Q5 kit (New England Biolabs; USA) and custom-synthesized primers (Supporting Information Table S3); the resulting plasmid was pBAS23. The presence of the desired codon change and absence of other undesired alterations in the 2.6-kb genomic region present in pBAS23 was verified by DNA sequence analysis.

To construct a deletion of the *R. pomeroyi* chromosomal *ntrYX* gene cluster, 600-bp fragments located upstream and downstream of the respective genomic area (Fig. 8A) were amplified by PCR using custom synthesized primers (Supporting Information Table S3). A DNA fragment encompassing a gentamycin resistance cassette was amplified from plasmid p34S_Gm (Dennis and Zylstra, 1998). Using the Gibson assembly procedure (Gibson *et al.*, 2009), the three DNA fragments were cloned into the linearized (by cutting with EcoRI and XbaI) suicide vector pK18mobsacB (Kvitko and Collmer, 2011), which confers resistance to kanamycin. The resulting plasmid was pBAS41 and carries the Δ(*ntrXY::Gm*)1 mutation.

Construction of R. pomeroyi chromosomal gene disruption mutants

Plasmid pBAS41 [Δ(*ntrYX::Gm*)1] was conjugated via triparental mating by mixing the *E. coli* strain PRK2015 (pRK2013) [Kan^{R}] (Figurski and Helinski, 1979), and DH5α (pBAS41) [Kan^{R} Gm^{R}] and the Rif^{R} *R. pomeroyi* recipient strain J470 as detained previously (Schulz *et al.*, 2017). *R. pomeroyi* J470 trans-conjugants that had received plasmid pBAS41 were selected on 1/2 YTSS agar plates containing the antibiotics rifampicin and gentamycin; the integration of the Δ(*ntrYX::Gm*)1 gene disruption mutation into the chromosome via a double homologous recombination event was selected for by including 10% saccharose into the agar plates (Schulz *et al.*, 2017). The resulting Kan^{S} Gm^{R} trans-conjugates were evaluated via PCR for the presence of the chromosomal Δ(*ntrYX::Gm*) deletion/insertion mutation and loss of the wild-type *ntrYX* genes using chromosomal DNA as the template and DNA primers listed in Supporting Information Table S3; these hybridize to genomic regions flanking the *ntrYX* gene cluster. The Δ(*ntrYX::Gm*)1 mutation removes the overlapping genes *ntrX* and *ntrY* (3.695 bp) from the genome of *R. pomeroyi* strain J470 (Fig. 8A), with one deletion endpoint beginning at the GTG start codon of *ntrY* and the second deletion junction ending with the TAA stop codon of *ntrX*. This strain was named ASR9 (Supporting Information Table S1).

Mutagenesis with the EZ-Tn5 transposon and DNA-sequence analysis of specific chromosomal insertion sites

We used the EZ-Tn*5* [R6Kγori/KAN-2] transposition system (Epicentre, Madison, USA) to mutagenize *R. pomeroyi* DSS-3. All reagents required for these experiments were contained in the kit provided by the supplier. The EZ-Tn*5* [R6Kγori/KAN-2] transposon, along with a hyperactive Tn*5* transposase and a type I restriction enzyme inhibitor (both purchased from Epicentre) were electroporated into *R. pomeroyi* DSS-3 cells (puls: 1.6 kV, 200 Ω and 25 μF) using the Gene Pulser Xcell system (Biorad, Munich, Germany). The transformed cells were then carefully re-suspended (at 30°C) into 1 ml prewarmed basal minimal medium and incubated for 3 h at 30°C on a shaker. Portions of 20-μl cell suspension were subsequently plated on basal medium agar plates containing 120 μg ml^{-1} of kanamycin to select for chromosomal EZ-Tn*5* [R6Kγori/KAN-2] transposition insertions; the selective agar plates were incubated at 30°C for 7–14 days. To search for transposon insertions affecting the use of ectoine as a nutrient, colonies that had grown on the selection plates were replica plated onto basal medium agar plates containing 15 mM ectoine as sole carbon and nitrogen source and onto basal medium agar plates containing glucose (28 mM) and NH_4Cl (56 mM) as carbon and nitrogen sources. These replica plates were then incubated for 10 days at 30°C. From a collection of about 21 000 colonies with EZ-Tn*5* [R6Kγori/KAN-2] transposon insertions, three candidates with a growth defect in the use of ectoine were identified. These were purified by re-streaking on basal medium agar plates containing glucose, NH_4Cl and kanamycin and were subsequently tested for their inability to exploit ectoine as a nutrient on basal medium agar plates. The insertion site of the Tn*5* [R6Kγori/KAN-2] transposon in these strains was then determined either by RACE PCR with primers (purchased from Epicentre) listed in Supporting Information Table S3 or via rescue cloning. For this latter approach, chromosomal DNA from the Tn*5* [R6Kγori/KAN-2] transposon insertion strains was prepared, cleaved with EcoR1, re-ligated, and transformed into TransforMax™ EC100D™ *pir*$^{+}$ *E. coli* cells (Epicentre). The only plasmid able to replicate in these cells from the entire restriction/ligation mixture is the Tn*5* [R6Kγori/KAN-2] transposon along with its flanking genomic DNA sequences since the mini-transposon carries the R6Kγori region that is only functional in a *pir*$^{+}$ strain (e.g., not in *R. pomeroyi*). It establishes itself in the TransforMax™ EC100D™ *pir*$^{+}$ *E. coli* cells as a low-copy-number plasmid that can be selected for by plating the transformed cells on LB-agar plates containing 120 μg ml^{-1} kanamycin. DNA of these plasmids were prepared and the *R. pomeroyi* DNA sequences flanking the Tn*5* [R6Kγori/KAN-2] transposon insertion site were determined using primers (Supporting Information Table S3) provided in the EZ-Tn*5* [R6Kγori/KAN-2] transposition kit (Epicentre).

β-galactosidase enzyme activity measurements

R. pomeroyi strains carrying plasmids containing *lacZ* reporter genes were grown overnight in a basal medium containing glucose (28 mM) and NH_4Cl (200 mM) as the carbon and nitrogen source. These precultures were then used to inoculate (with maximally 500 μl) the main culture (25-ml) to an optical density (OD_{578}) of about 0.1. When ectoine or hydroxyectoine was used as sole carbon- or nitrogen sources by *R. pomeroyi*, they were separately provided at a concentration of 28 mM. The cultures were grown at 30°C until they had reached an OD_{578} of about 1. The cells were collected by

centrifugation (13 000 rpm for 10 min), and processed for β-galactosidase activity assays with ONPG as the chromogenic substrate (Miller, 1972). β-galactosidase enzyme activity is expressed as Miller Units (MU). A strain harbouring the promoterless *lacZ* fusion vector pBIO1878 (Todd *et al*., 2012) that was used to construct the reporter fusions used in this study yields ß-galactosidase background values between 2 and 5 MU (Schulz *et al*., 2017).

Overproduction, purification and ligand-binding assays with EnuR

For overproduction of EnuR-*Strep*-tag-II and EnuR*-*Strep*-tag-II recombinant proteins, cells of the *E. coli* B strain BL21 (DE3) were transformed with the appropriate overproduction plasmids [pBAS3 (*enuR*$^+$; pBAS17 (*enuR**)] (Supporting Information Table S2) that allow the expression of the *enuR* gene under the control of the *tet* promoter, a system (IBA, Germany) that is controlled by the anhydrotetracycline (AHT)-responsive TetR repressor (Schulz *et al*., 2017). The plasmid-containing *E. coli* cells were grown at 37°C in MMA containing 0.5% casamino acids until the OD_{578} reached 0.6 and the *tet*-promoter/TetR mediated overexpression of plasmid-encoded *enuR/enuR** genes was triggered by adding the TetR inducer AHT (final concentration: 0.2 μg ml^{-1}) to the growth medium. The growth temperature of the cultures was reduced to 35°C and they were subsequently incubated for additional two hours to allow overproduction of the recombinant EnuR and EnuR* proteins. Cells were then harvested by centrifugation, lysed and the EnuR and EnuR* proteins were purified from the cell extracts via affinity chromatography on a Strep-Tactin Superflow column as described (Schulz *et al*., 2017).

Ligand binding assays with the purified EnuR and EnuR* proteins were carried out by microscale thermophoresis (MST) (Duhr and Braun, 2006; Wienken *et al*., 2010). All experiments were performed on a Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) at 21°C (red LED power was set to 80% and infrared laser power to 70%). The buffer of the purified EnuR and EnuR* [in 100 mM Tris- HCl (pH 7.5), 150 mM NaCl] was first exchanged with the labelling buffer of the Monolith NTTM Protein Labeling Kit RED (NanoTemper) to avoid interference of the labelling reactions with free amines in the buffer solution. Subsequent to labelling of EnuR and EnuR* (20 μM each) with the dye NT 647 (according to the suppliers reaction protocol), the EnuR/EnuR* proteins were re-buffered into a solution buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.2% Tween. 200 nM EnuR or EnuR* was titrated with ectoine or hydroxyectoine starting from a concentration of 10 mM. Both proteins were also titrated with *N*-(α)-ADABA or *N*-(γ)-ADABA (starting from 65 μM and 1 mM, respectively). Additionally, 200 nM EnuR was titrated with DABA with a starting concentration of 50 mM. At least six independent MST experiments per ligand and type of EnuR protein were recorded at 680 nm and analysed using NanoTemper Analysis 1.2.009 and Origin8G software suits.

Electrophoretic mobility shift assays

Fluorescently labelled DNA fragments for electrophoretic mobility shift assays (EMSA) were generated by PCR from genomic DNA of *R. pomeroyi* DSS-3. For the *uehA* regulatory region, primers L1_rev-dye and L2_fw (Supporting Information Table S3) were used to generate a 251-bp fragment containing the putative *uehA* promoter. A 278-bp fragment of the *uehA*-coding region was generated using primers L5_rev_dye and L4_fw (Supporting Information Table S3). The DNA primers L1_rev_dye and L5_rev_dye were 5'-labelled with the Dyomics 781 fluorescent dye (Microsynth AG, Balgach, Switzerland). Binding reactions between the DNA fragments (0.5 pmol) and various concentrations of the purified EnuR protein were performed in buffer A (20 mM phosphate [pH 7.0], 1 mM dithiothreitol, 5 mM $MgCl_2$, 50 mM KCl, 15 μg/ml bovine serum albumin, 50 μg/ml salmon sperm DNA, and 5% [vol/vol] glycerol, 0.1% Tween20) in a total volume of 20 μl. After incubation of the reaction mixture, the samples were loaded onto a native 5% polyacrylamide gel and electorphoretically separated at 110 V for 45 min. EnuR:DNA-interactions were detected using an Odyssey FC Imaging System (LI-COR Biosciences, Linoln, USA).

Database searches for potential microbial ectoine consumers and phylogenetic analysis of the EnuR, AsnC and NtrYX regulatory proteins

Searches for orthologues of the *R. pomeroyi* DSS-3 EutD protein (accession number: AAV94440.1) (Moran *et al*., 2004) had previously been conducted 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). The taxonomic affiliation of the potential hydroxyectoine/ectoine consumers had been analysed and visualized via the Interactive Tree of Life iTOL web-tool (http://itol.embl.de/) (Letunic and Bork, 2011). This curated dataset comprised 539 entries and 456 of these possessed a *enuR*-related gene in the immediate vicinity of the hydroxyectoine/ectoine import and catabolic gene cluster (Schulz *et al*., 2017). This data set was newly further searched for the presence of an *asnC*-type gene positioned within the hydroxyectoine/ectoine import and catabolic gene clusters. Furthermore, the genome sequences of the 539 potential hydroxyectoine/ectoine consumers were again queried through a BLAST search for the presence of NtrXY-type two-component regulatory systems. Along with EnuR, the presence of AsnC and NtrXY proteins was then projected onto the previously reported EutD-derived phylogenetic tree (Schulz *et al*., 2017) (Supporting Information Fig. S3).

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CONFLICT OF INTEREST

The authors declare that they have no financial conflict of interest with regard to the data presented in this study.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table S1. *Ruegeria pomeroyi* strains used in this study.
Table S2. Plasmids used in this study.
Table S3. Primers used in this study.
Fig. S1. Ligand binding by the wild-type EnuR protein and its mutant EnuR* derivative as assessed by microscale thermophoresis. Purified EnuR protein (200 nM) was titrated with increasing concentrations of (A) ectoine, (B) hydroxyectoine, and (C) γ-L-ADABA. (D) Purified mutant EnuR* protein (200 nM) was titrated with increasing concentrations of α-L-ADABA.
Fig. S2. Growth curves of rifampicin-resistant *R. pomeroyi* J470 mutant derivatives defective in various ectoine catabolic genes. Cultures were grown in basal minimal media containing either ectoine (squares) or hydroxyectoine (circles) as the sole carbon and nitrogen source, or glucose and NH_4Cl (rhombi) as carbon and nitrogen source respectively. Ectoine, hydroxyectoine, and glucose were present in these cultures at a concentration of 28 mM. NH_4Cl was added to a final concentration of 200 mM. (A) Growth of the *R. pomeryi* strain ASR8 [Δ(*eutD::Gm*)1] (B) Growth of the *R. pomeryi* strain ASR11 [Δ(*eutABC::Gm*)1].
Fig. S3. Taxonomic distribution of *enuR*, *asnC*, and *ntrYX* genes among microorganisms predicted to consume ectoines. An alignment of 539 amino acid sequences homologous to the EutD protein from *Ruegeria pomeroyi* DSS-3 has previously been used to construct a phylogenetic tree of presumed consumers of ectoines. The color code outlines the distribution of EutD-type proteins among the classes of the *Proteobacteria*. The presence of *enuR* or *asnC* genes in the direct vicinity (for *enuR*) or within (for *asnC*) hydroxyectoine/ectoine-transport and catabolic gene clusters are indicated by a pink pentagon and a purple circle respectively. The presence of a gene cluster (*ntrXY*) encoding the two-component NtrYX regulatory system in the genome sequences of the predicted consumers of ectoines is indicated by orange pentagons.
Fig. S4. Growth of rifampicin-resistant *R. pomeroyi* wild type J470 and its mutant derivative ASR9 which carries a deletionfor *ntrXY* (Δ*ntrXY*). Cells were grown on basal minimal medium agar plates containing ectoine, glycine betaine or choline either as a sole carbon, as a sole nitrogen or as a combined carbon and nitrogen source as indicated.