Guilty by association: importers, exporters and MscS-type mechanosensitive channels encoded in biosynthetic gene clusters for the stress-protectant ectoine

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ABSTRACT

Ectoine and its derivative hydroxyectoine are widely synthesized or imported by bacteria to fend-off the detrimental effects of high osmolarity on cellular hydration and growth. Genes that are connected to a particular physiological process are often found in the same genomic context. We exploited this feature in a comprehensive bioinformatical analysis of 1,103 ectoine biosynthetic gene clusters from *Bacteria* and *Archaea* through which we identified 415 *ect* operons that colocalize with genes encoding potential osmolyte transporters. These belong to various importer families. Focusing on the complex *ect* gene clusters of the alpha-proteobacteria *Hyphomonas neptunium* and *Novoshingobium sp*. LH128, we analyzed several transporters with respect to their substrate specificities through physiological, molecular and modelling approaches. Accordingly, we identified an MFS-type uptake system specific for ectoines (EctU) and a novel SSS-type ectoine/hydroxyectoine importer (EctI) with a broader substrate profile for osmostress protectants. Furthermore, some *ect* gene clusters encode a MscS/YbdG-type mechanosensitive channel protein whose functionality was assessed through down-shock assays. Moreover, our analysis identified the gene for the first putative ectoine/hydroxyectoine-specific efflux system (EctX), a member of the MFS superfamily. Our findings make substantial contributions to the understanding of the ecophysiology of ectoines, key players in microbial osmostress adjustment systems.

IMPORTANCE

Osmotic stress is a ubiquitously occurring environmental challenge that free-living microorganisms have to cope with. The accumulation of compatible solutes, physiologically compliant organic osmolytes, is a flexible adaptation strategy to prevent cellular dehydration, drop of turgor, and the secession of growth under osmotically unfavorable conditions. Ectoine and hydroxyectoine are major microbial osmostress protectants, and also serve as chemical chaperones to preserve the functioning of key cellular processes. Import of these solutes is an energetically favorable way to amass ectoines under osmotic stress conditions. Genes related to a particular metabolic process are often encoded in the same genomic context, a feature which offers the possibility to discover novel gene functions. By relying on such a "guilty by association approach" we discovered that many ectoine biosynthetic gene clusters encode transporters and mechanosensitive channels mediating fluxes of ectoines across the cytoplasmic membrane. Most notably is our discovery of the first ectoine/hydroxyectoine-specific efflux system.

KEYWORDS Osmotic stress, compatible solutes, importers, exporters, mechanosensitive channels, genomics

INTRODUCTION

Presumably the most important mechanism behind the evolutionarily success of bacteria is their ability to adapt flexible to a multitude of environmental challenges. Fluctuations in the external osmolarity impose considerable constraints onto the cell with respect to a physiologically appropriate degree of hydration, magnitude of turgor, level of molecular crowding, efficiency of growth, and of cellular integrity (Kempf & Bremer, 1998, Roeßler & Müller, 2001, Gunde-Cimerman *et al.*, 2018, Wood *et al.*, 2001). The high osmotic potential of the crowded cytoplasm and the bio-physical properties of the semipermeable cytoplasmic membrane trigger the influx of water into the cell and promote the build-up of an out-ward directed hydrostatic pressure, turgor (Bremer & Krämer, 2019, Wood, 2011, van den Berg *et al.*, 2017). Osmotically driven water fluxes at high osmolarity therefore threaten the cell with dehydration and drop of turgor to physiologically non-sustainable low values, or rupture under suddenly imposed low osmolarity surrounding due to a strong increase of turgor. Osmoregulation is thus a fundamental need for every free-living microbial cell (Bremer & Krämer, 2019, Wood, 2011, Booth, 2014).

Microorganisms lack active transport systems for water; hence, they have to rely on indirect mechanisms to scale and direct water fluxes when the cell is challenged by fluctuating environmental osmolarity. This is accomplished through an active management of the cytoplasmic solute pool (Bremer & Krämer, 2019, Wood, 2011). Upon exposure to high osmolarity, many bacteria transiently over-accumulate K^+ ions to promote water retention and entry. However, excess of this ion is subsequently largely substituted with physiologically compliant organic osmolytes, the so-called compatible solutes. This prevents the development of a long-lasting high ionic-strength cytoplasm without impairing the osmotic potential of this cellular compartment. The accumulation of function-preserving compatible solutes (Ignatova & Gierasch, 2006, Stadmiller *et al.*, 2017, Street *et al.*, 2006) can be achieved either through their energetically expensive biosynthesis, or their energetically more favorable import from environmental sources (Bremer & Krämer, 2019, Wood, 2011, Oren, 1999). Once the external osmolarity suddenly drops, excess water entry is counteracted by the cell by rapidly, but non-specifically, jettison prior accumulated compatible solutes, ions, and metabolites via the transiently opening of mechanosensitive channels. This emergency reaction avoids cell rupture by rapidly reducing the magnitude of turgor (Booth, 2014, Cox *et al.*, 2018).

Ectoine (Galinski *et al.*, 1985) and its derivative hydroxyectoine (Inbar & Lapidot, 1988) (Fig. 1A) are important representatives of the compatible solutes (da Costa *et al.*, 1998) and are widely used as osmostress protectants in microorganisms (Pastor *et al.*, 2010, Czech *et al.*, 2018a). The enzymes mediating their synthesis are now rather well understood, both biochemically and structurally (Hermann *et al.*, 2020). In addition to serving as compounds whose accumulation can promote cellular hydration, ectoines also optimize the solvent properties of the cytoplasm by providing chemical

chaperon activities for various cell constituents (Zaccai *et al.*, 2016, Barth *et al.*, 2000, Lippert & Galinski, 1992, Hahn *et al.*, 2017, Harishchandra *et al.*, 2011, Tanne *et al.*, 2014).

Ectoines are brought into the environment when bacterial producer cells lyse (Welsh, 2000), through the transient opening of mechanosensitive channels (Booth, 2014, Hoffmann *et al.*, 2008), through active excretion (Grammann *et al.*, 2002, Schubert *et al.*, 2007, Jebbar *et al.*, 1992, Czech *et al.*, 2016, Czech *et al.*, 2018b, Vandrich *et al.*, 2020), and through their production by some protists and microalgae in marine ecosystems (Weinisch *et al.*, 2019, Fenizia *et al.*, 2020, Nowinski & Moran, 2021). Hence, osmotically stressed bacterial cells can take advantage of environmental ectoines (Mosier *et al.*, 2013, Bouskill *et al.*, 2016, Warren, 2022) through import either by using them as stress protectants or as nutrients (Hermann *et al.*, 2020).

To date, ectoine importers belonging to four major transporter families have been identified: (i) binding-protein dependent ATP binding cassette (ABC) systems, (ii) tripartite ATP-independent periplasmic transporters (TRAP-T), (iii) betaine-choline-carnitine-transporters (BCCT), and (iv) systems of major facilitator superfamily (MFS). In a limited number of instances, genes for ectoine importers were found to be associated with ectoine/hydroxyectoine biosynthetic operons (Richter *et al.*, 2019, Gregory & Boyd, 2021, Leon *et al.*, 2018, Frikha-Dammak *et al.*, 2021). A few of these osmotically inducible gene clusters even comprise a gene for an MscS-type mechanosensitive channel whose function is important for managing the cell's transition from high to low osmolarity surroundings (Widderich *et al.*, 2016, Booth, 2014, Czech *et al.*, 2016).

Genes that are connected to a particular physiological task often cluster in microbial genomes. Pathway prediction approaches that combine database searches with information gleaned from protein crystal structures can inform the annotation and functional characterization of unknown physiological traits (Zhao *et al.*, 2013, Kumar *et al.*, 2014). Using this *guilty by association approach*, we found that a large number of ectoine/hydroxyectoine biosynthetic gene clusters either comprise, or are associated with, genes functionally related to various families of previously characterized ectoine/hydroxyectoine import systems. Building on this information, we physiologically characterized two new transport systems for ectoines, members of the MFS and SSS (sodium solute symporter) families, that differed in their substrate profile for compatible solutes. We also found a number of *ect* biosynthetic gene clusters that contained a gene for an MscS/YbdG-type mechanosensitive channel protein. We assessed the functionality of one of these presumed channels through osmotic down-shock experiments. Most interesting, we provide physiological evidence for the identification of the first ectoine/hydroxyectoine-specific secretion system in bacteria and discuss its potential role(s) in the context of the cell's adjustment systems to persistent or fluctuations in environmental osmolarity and with respect to the behavior of cells in microbial communities.

Results

Identification of potential importers and exporters for ectoines through bioinformatics

Ectoine biosynthetic genes are typically organized in an evolutionarily conserved and osmotically inducible operon (*ectABC*), which might also comprise the gene for the ectoine hydroxylase (EctD) (Bursy *et al.*, 2007, Argandona *et al.*, 2021), and/or that for a specialized aspartokinase (Ask_Ect), an enzyme enhancing the synthesis of the ectoine biosynthetic precursor L-aspartate-β-semialdehyde (Stöveken *et al.*, 2011, Hermann *et al.*, 2020). These gene clusters are also often associated with a gene encoding a MarR-type repressor (EctR) controlling *ect* gene transcription (Mustakhimov *et al.*, 2010). The ectoine synthase (EctC) is the signature enzyme of the ectoine biosynthetic pathway (Hermann *et al.*, 2020) but solitary EctC-related proteins exist that seem not to be involved in ectoine biosynthesis (Czech *et al.*, 2018a, Kurz *et al.*, 2010). We used the biochemically and structurally characterized EctC ectoine synthase from *Paenibacillus lautus* (Czech *et al.*, 2019a) as the search query to update previous reported data base searches for ectoine biosynthetic gene clusters (Hermann *et al.*, 2020, Czech *et al.*, 2018a, Mais *et al.*, 2020). To this end we used a curated dataset of 14,201 completely sequenced prokaryotic genomes represented in the Integrated Microbial Genomes & Microbiomes (IMG/M) database (Chen *et al.*, 2021). In 1,089 of these genomes, we found 1,103 ectoine/hydroxyectoine biosynthetic gene clusters; 1,091 of these were present in 1,077 genomes of members of the *Bacteria* and 12 *ect* gene clusters were from derived from *Archaea*. We note in this context that 14 bacterial genome sequences contain multiple ectoine biosynthetic gene clusters, many of which represent species of the genus *Streptomyces*.

Using this dataset, we manually inspected the genome context of each *ect* gene clusters for genes that potentially could encode transporters. We used for this analysis computational gene annotation resources provided through the IMG/M database (Chen *et al.*, 2021) and we found 523 genes for putative transporter systems that were either embedded in the inspected *ect* gene clusters, or were juxtapositioned to them. Collectively, these putative transporter genes were found in 415 *ect* gene clusters as some of them possess multiple predicted transporter genes. Hence, about 37% of the 1,103 inspected ectoine/hydroxyectoine biosynthetic gene clusters harbor, or are associated with, genes for predicted transporters.

A more detailed analysis of the types of these transport systems revealed that they mostly belong to five different major transporter families. They represent either ABC, TRAP-T, BCCT, MFS, and SSS families, all of which (with the exception of the SSS) comprise previously functionally characterized transport systems with the ability to import ectoines. The abundance of the various transporter families and their amino acid sequence identities to functionally characterized osmolyte importer systems are summarized in Table 1. Members of the ABC transporter family (220 representatives) and MFS-type transporters (173 representatives) were most frequently associated with ectoine

biosynthetic gene clusters. Among the MFS transporters, predicted importers (117 representatives) and predicted export systems (56 representatives) were present, as the former group is related to the proton coupled osmolyte importer ProP from *E. coli* capable to import ectoines (MacMillan *et al.*, 1999, Jebbar *et al.*, 1992), while the latter group is related to the multi-drug exporters YajR, MdfA and YcaD, respectively (Heng *et al.*, 2015, Jiang *et al.*, 2013, Pade & Hagemann, 2014, Law & Alegre, 2018). Among the ABC transporters, systems related to ProU, Ehu, and OpuB/OpuC were identified. This classification relied on crystal structures of their extracellular substrate binding proteins and their experimentally assessed substrate profile (Schiefner *et al.*, 2004, Hanekop *et al.*, 2007, Horn *et al.*, 2006, Du *et al.*, 2011, Jebbar *et al.*, 1992, Jebbar *et al.*, 1997). Members of the BCCT, SSS and TRAP-T systems collectively represent 20% of the overall transporter families in our dataset (Table 1). Exemplary genetic organizations of ectoine gene clusters associated with genes encoding transporter are shown in Fig. 1B. As documented in Supplementary Fig. 1, one should note that a considerable variation in the gene organization of transporter genes associated with *ect* clusters exists.

We also found *ect* biosynthesis gene clusters that contained a gene encoding putative a mechanosensitive channel protein (23 representatives) (Table 1) that are related to YbdG-type systems, members of the wider MscS family of channels (Edwards *et al.*, 2012, Schumann *et al.*, 2010, Amemiya *et al.*, 2019). Mechanosensitive channels of the MscL, MscS and MscM families serve as safety valves to prevent lysis when bacterial cells are subjected to sudden osmotic down-shocks (Booth, 2014, Cox *et al.*, 2018). The presence of genes encoding members of MscS-type mechanosensitive channels juxtapositioned to ectoine biosynthetic genes is somewhat surprising but not without precedent (Widderich *et al.*, 2016). Hence, in *msc*-containing *ect* gene clusters, genes for the cellular adjustment to high osmolarity environments colocalize with a gene required for managing the transition from high to low osmolarity surroundings.

Modelling of ectoine/hydroxyectoine substrate binding proteins from Ehu-, ProX- and OpuB/OpuC-type ABC systems, the Tea-type TRAP transporter and of the BCCT-type importer

To further support our bioinformatic assignment of putative ectoine/hydroxyectoine transporter genes associated with *ect* biosynthetic gene clusters, we performed modelling and docking studies. High-affinity substrate binding proteins from either ABC transporters or TRAP-T systems have been crystallized in the presence of ectoine and hydroxyectoine (Hanekop *et al.*, 2007, Kuhlmann & Bremer, 2002, Lecher *et al.*, 2009, Du *et al.*, 2011). EhuB is the substrate binding protein of the EhuABCD ABC system from *Sinorhizobium meliloti*, a transporter for ectoines when they are used as nutrients (Hanekop *et al.*, 2007, Jebbar *et al.*, 2005). However, Ehu-type transporters have also been previously found juxtapositioned to ectoine/hydroxyectoine biosynthetic gene clusters (Richter *et al.*, 2019, Frikha-Dammak *et al.*, 2021), implying a role for these transporters for the acquisition of ectoines as

osmostress protectants. Compared with the *S. meliloti* EhuB protein, the 134 EhuB substrate binding proteins in our dataset (Table 1) had a degree of amino acid sequence identity ranging between 74% and 33%. The amino acids involved in ectoine/hydroxyectoine binding by the *S. meliloti* EhuB protein (Hanekop *et al.*, 2007) are highly conserved and this is documented in an abbreviated amino acid sequence alignment of ten EhuB-type proteins from our dataset in Supplementary Fig. 2. *in silico* modelling and structure comparison using the EhuB-type proteins of *Mesorhizobium loti* (74% identity to the EhuB protein from *S. meliloti*) and of *Algicoccus marinus* HZ20 (33% identity to the EhuB protein from *S. meliloti*) as exemplary representatives revealed a ligand binding pocket for ectoines with an architecture highly similar to that revealed through structural analysis of the *S. meliloti* EhuB substrate binding protein (Hanekop *et al.*, 2007) (Supplementary Fig. 2).

Our dataset contained representatives of the binding-protein-dependent ABC-type transporters ProU from *E. coli* and OpuB/OpuC from *Bacillus subtilis*. The substrate binding proteins (ProX, OpuBC, OpuCC) of these transporters have all previously been crystallized (Schiefner *et al.*, 2004, Du *et al.*, 2011, Pittelkow *et al.*, 2011). Modelling and sequence analysis of the OpuBC-type substrate binding protein from *Virgibacillus halodenitrificans* revealed a similar overall fold and high sequence similarity to the structurally characterized OpuCC protein from *B. subtilis* in complex with ectoine (PDB ID: 3PPR) (Du *et al.*, 2011). Overlay and comparison of the substrate binding site shows that the residues crucial for ectoine binding are conserved (Supplementary Fig. 3). Different molecular determinants mediate recognition of glycine betaine and ectoine in the OpuCC substrate binding protein of the promiscuous OpuC ABC transporter of *B. subtilis* (Du *et al.*, 2011, Hoffmann & Bremer, 2017).

A homolog of a third compatible solute ABC-type transporter, ProU from *E. coli,* was found to be encoded in the *ect* gene neighborhood (e.g., of *Vibrio anguillarum*). *In silico* modelling analysis revealed an overall fold and conservation of the crucial ligand binding residues when the substrate binding proteins are compared to the well characterized ProX protein of the *E. coli* ProU transporter. The corresponding ProX binding protein has been crystalized in complex with glycine betaine (PDB ID: 1R9L) (Schiefner *et al.*, 2004) but not in the presence of ectoine, although the ProU system can mediate ectoine/hydroxyectoine import (Jebbar *et al.*, 1992, MacMillan *et al.*, 1999) (Supplementary Fig. 4).

Substrate-binding-protein dependent TRAP-type transporters are a minority (8 out of 523) of the import systems encoded in the vicinity of ectoine/hydroxyectoine biosynthetic gene clusters (Table 1, Fig. 1). UehA is the substrate binding protein of a TRAP-T system (UehABC) used by the marine bacterium *Ruegeria pomeroyi* DSS3 to acquire ectoines as nutrients (Lecher *et al.*, 2009). In contrast, the TeaA substrate binding protein of the TeaABC TRAP-T system from the salt-tolerant bacterium *Halomonas elongata* is involved in the acquisition of ectoines as osmostress protectants (Grammann *et al.*, 2002). Despite these differences in physiological function and transcriptional regulation of the

corresponding transporter genes, the ligand-binding sites of the UehA and TeaA substrate binding proteins are superimposable (Lecher *et al.*, 2009, Kuhlmann *et al.*, 2008). TeaA has been crystalized in the presence of either ectoine or hydroxyectoine, while an UehA crystal structure was obtained only in complex with ectoine. Compared with the *H. elongata* TeaA protein (Kuhlmann *et al.*, 2008, Grammann *et al.*, 2002), the eight TeaA-type substrate binding proteins in our dataset (Table 2) had a degree of amino acid sequence identity ranging between 27% and 33%. Their amino acid sequence alignment to the *H. elongata* TeaA protein revealed a high degree of amino acid conservation of those residues used to coordinate the ectoine and hydroxyectoine ligands within the TeaA substrate binding site (Supplementary Fig. 5). Not surprisingly, *in silico* modeling and structural comparison with the TeaA-type protein from the marine bacterium *Alcanivorax borkumensis* (33% identity to the TeaA protein from *H. elongata*) revealed an overall fold and architecture closely resembling those of the *H. elongata* TeaA protein in complex with either ectoine or hydroxyectoine (Supplementary Fig. 5).

The BCCT-family of single-component carriers contains a number of ectoine/hydroxyectoine transporters (Ziegler *et al.*, 2010, Gregory & Boyd, 2021). Two of its members have been crystalized: BetP from *Corynebacterium glutamicum* in the presence of glycine betaine and CaiT from *E.coli* in the presence of carnitine (Ressl *et al.*, 2009, Schulze *et al.*, 2010), but no crystal structure in complex with ectoine is available. Modelling of the BCCT-type transporter from *Vibrio alginolyticus* revealed an overall fold that largely resembles the crystal structure of the *C. glutamicum* BetP transporter (PDB ID: 2WIT). This model had a root means square deviation (RMSD) value of 1.07 Å (Supplementary Fig. 6). Docking analysis of ectoine and hydroxyectoine in the modeled *V. alginolyticus* BCCT-system resulted in a placement of both molecules that is superimposable to the glycine betaine molecule present in the crystal structure of BetP (Supplementary Fig. 6). Sequence alignment revealed a high conservation of those residues involved in ligand binding and coordination of the co-transported Na^+ ions (Ressl *et al.*, 2009). In an independent study, Gregory et al. (2020) modelled the overall fold of an ectoine transporter (BccT1) from *Vibrio parahaemolyticus* and derived a solution for the ectoine binding site (Gregory *et al.*, 2020) similar to the one that we predict for the *V. alginolyticus* BCCT ectoine/hydroxyectoine import system (Supplementary Fig. 6).

Novoshingobium sp. LH128 and Hyphomonas neptunium accumulate hydroxyectoine in response to salt stress

For our further analysis of putative ectoine/hydroxyectoine transport systems, we focused on two particularly complex *ect* gene clusters that were present in two alpha-proteobacteria, *Novoshingobium* sp. LH128, and *Hyphomonas neptunium*, respectively (Fig. 2A,D). These microorganisms live in different habitats, as *Novoshingobium* sp. LH128 is a soil bacterium (Bastiaens *et al.*, 2000), while *H. neptunium* strives in marine ecosystems (Badger *et al.*, 2005). The *ect* gene clusters from these two

microorganisms attracted our particular interest because they contain genes encoding three different types of predicted transporters; an MFS-type importer, an SSS-type importer, and a putative MFS-type exporter. Moreover, the *ect* gene cluster of *Novoshingobium* sp. LH128 additionally harbors a gene for a YbdG-type mechanosensitive channel protein (Schumann *et al.*, 2010, Amemiya *et al.*, 2019, Edwards *et al.*, 2012) (Fig. 2A,D). Both *Novoshingobium* sp. LH128 and *H. neptunium* contain juxtapositioned to their ectoine biosynthetic gene cluster (*ectABC*), genes for the ectoine hydroxylase (*ectD*) (Bursy *et al.*, 2007), and for the specialized aspartokinase (*ask_ect*) (Stöveken *et al.*, 2011) involved in the synthesis of the ectoine biosynthetic precursor L-aspartate-ß-semialdehyde (Hermann *et al.*, 2020). In addition, a divergently transcribed gene for the MarR-type regulator EctR (Mustakhimov *et al.*, 2010) was present as well (Fig. 2A,D).

To test the functionality of the above described ectoine/hydroxyectoine biosynthetic gene clusters (Fig. 2A, and D) and to explore the salt tolerance of *H. neptunium* and *Novosphingobium* sp. LH128, we cultivated cells of these bacteria using media with increasing NaCl concentrations (Fig. 3A,B). After 24 h of incubation, we measured the growth yield of the cultures (at OD_{578}), harvested cells by centrifugation and extracted the produced ectoines for HPLC analysis. *H. neptunium* was grown in marine broth, a rich medium with a measured osmolarity of 920 mOsm kg^{-1}. As expected, increases in the salinity of the growth medium concomitantly reduced the growth yield of the cultures. Growth was in essence prevented when 0.9 M NaCl was added to the cultures (Fig. 3A), thereby raising the overall osmolarity of the medium to 2,860 mOsm kg^{-1}. *Novosphingobium* sp. LH128 was cultivated in a chemically fully defined medium [Spizizen minimal medium (SMM)] with glucose as the carbon source. In contrast to *H. neptunium*, *Novosphingobium* sp. LH128 was only able to grow in a rather restricted range of salinities; from 0 M NaCl (377 mOsm kg^{-1}) to 0.5 M NaCl (1,340 mOsm kg^{-1}) NaCl (Fig. 3B).

Synthesis of hydroxyectoine by the ectoine hydroxylase EctD is dependent on the prior production of ectoine (Hermann *et al.*, 2020, Höppner *et al.*, 2014). Microorganisms that possess EctD can either produce a mixture of the two ectoines, or synthesize exclusively hydroxyectoine (Pastor *et al.*, 2010, Czech *et al.*, 2018a, Argandona *et al.*, 2021). We found that *H. neptunium* and *Novosphingobium* sp. LH128 synthesized exclusively hydroxyectoine when exposed on a sustained basis to high-salinity surroundings (Fig. 3C,D). In both bacteria, the cellular pools of this compatible solute were linearly dependent on the salt concentration of the growth medium (Fig. 3C,D). Such a proportional ectoine production and corresponding transcriptional control of the *ect* promoter in response to step-wise increases in the external salinity has been observed before in other microorganisms (Kuhlmann & Bremer, 2002, Czech *et al.*, 2018b, Calderon *et al.*, 2004). Collectively, these findings suggest that *H. neptunium* and *Novosphingobium* sp. LH128 must possess mechanisms to detect incremental increases in the external salinity and transform this environmental cue into a

genetic/physiological signal allowing the precise adjustment of their osmostress-protective cytoplasmic ectoine/hydroxyectoine pools.

Functional annotation of the transporter genes contained in the ect gene clusters from Novoshingobium sp. LH128 and H. neptunium

Bioinformatic analysis of the two transporter types whose genes are present downstream of the *ect* genes in *Novosphingobium* sp. LH128 (Fig. 2A) showed that they belong to the Major Facilitator Superfamily (MFS). The first transporter positioned downstream of the gene encoding the specialized aspartokinase Ask_Ect (Reshetnikov *et al.*, 2006, Stöveken *et al.*, 2011) exhibits a sequence identity of 26.1% to the multidrug exporter MdfA and 27.2% to the putative exporter YcaD from *E. coli* (Heng *et al.*, 2015, Pao *et al.*, 1998). This suggests that this gene encodes an export system; hence, we refer in the following to this putative ectoine/hydroxyectoine exporter as EctX and to its structural gene as *ectX* (*ect*oine *e*xport) (Fig. 2A). The second MFS-type transporter possesses sequence identities of 42.1% to the osmolyte transporter ProP from *E. coli* (Ozturk *et al.*, 2020) and 44.4% to the compatible solute uptake system OusA from *Erwinia chrysanthemi* (Gouesbet *et al.*, 1996). We named this putative uptake system EctU (for ectoine uptake) (Fig. 2A). The next open reading frame is located 110 bp downstream of *ectU* and can be annotated as a gene for an YbdG-type mechanosensitive channel protein with 22.7% and 54.2% sequence identity to the *E. coli* MscS and YbdG proteins (Edwards *et al.*, 2012, Schumann *et al.*, 2010, Amemiya *et al.*, 2019), respectively (Fig. 2A). Several genes with uncertain physiological function directly follow the *mscS* gene of *Novosphingobium* sp. LH128 (Fig. 2A). In the *H. neptunium ect* gene cluster, an *ectX*-type gene is present as well (Fig. 2D). This particular EctX protein exhibits a sequence identity of 34.8% to the MdfA protein and 27.6% to the YcaD protein from *E. coli* (Heng *et al.*, 2015, Pao *et al.*, 1998).

The second transporter encoded by the *H. neptunium* gene cluster (Fig. 2B) belongs to the Sodium Solute Superfamily (SSS) of transporters and possesses 24.6% sequence identity to the sugar transporter SglT from *Vibrio parahaemolyticus* (Watanabe *et al.*, 2010), and 29% to the osmoregulated L-proline transporter OpuE from *Bacillus subtilis* (von Blohn *et al.*, 1997), hinting that it might serve as an uptake system for compatible solutes. We refer in the following to this putative ectoine/hydroxyectoine importer from *H. neptunium* as EctI and to its structural genes as *ectI* (*ect*oine *i*mport). The *ectI* gene is followed on the *H. neptunium* chromosome by a 296 bp intergenic region, and then by a gene encoding a putative RND family efflux system (Fig. 2D). The *ect* gene cluster of *H. neptunium* lacks gene a for a YbdG-type mechanosensitive channel protein.

The intergenic regions in the extended *ect* gene clusters of *Novosphingobium* sp. LH128 and *H. neptunium* are short, indicating that both of them are transcribed as an operon. We used RT-PCR to assess this hypothesis and found that the extended *ect* gene clusters of both microorganisms are

actually co-transcribed (Fig. 2B and E). In *Novosphingobium* sp. LH128 the transcript extends from *ectA* to *mscS*, a poly-cistronic mRNA with a calculated length of 8,522 bp. The three genes following *mscS* are co-transcribed as well but are not part of the *ect* transcript (Fig. 2B). The *ect* gene clusters from *H. neptunium* is also transcribed as a poly-cistronic operon with a calculated length of the mRNA of 7,535 bp; it extends from *ectA* to *ectI*. The gene for the RND extrusion system is not part of this mRNA (Fig. 2D and E).

Osmotic upregulation of the Novoshingobium sp. LH128 and H. neptunium ect gene clusters

In line with the finely tuned salt-responsive accumulation of ectoines, the expression of the underlying biosynthetic genes is typically up-regulated when cells are confronted with elevated external salt concentrations (Czech *et al.*, 2018a). We performed quantitative real-time PCR to test if the expression of the ectoine/hydroxyectoine biosynthetic genes in *H. neptunium* and *Novosphingobium* sp. LH128 are also responsive to osmotic stress. To this end, we grew cells of these bacteria both in the absence and the presence of NaCl; 0.5 M NaCl in the case of *H. neptunium* and 0.6 M NaCl for *Novosphingobium* sp. LH128. Total RNA of these cultures was subsequently isolated and one-step qRT-PCR experiments were conducted in which short fragments in the genes *ectA*, *ectD*, *ectX*, *ectI/ectU* were amplified after reverse transcription of the mRNA to cDNA. For *Novosphingobium* sp. LH128 we also assed the transcript of the *ybdG-type* gene (Fig. 2A and D). Transcripts of the *gyrB* genes of *H. neptunium* (WP_011645581.1) and of *Novosphingobium* (EJU15051.1) were used as a reference to benchmark the levels of the *ectA*, *ectD*, *ectX*, *ectI/ectU* and *ybdG* transcripts. The level of *gyrB* transcripts changed less than two-fold when the mRNA levels of salt-stressed cells and non-stressed *H. neptunium* and *Novosphingobium* sp. LH128 cells were compared. Salt-induction of transcription could be shown for the *ectA*, *ectD*, *ectX* and *ectU* genes from *Novosphingobium* sp. LH128 where the transcript levels increased on average by about 6-fold under osmotic stress conditions (Fig. 2C). The levels of the *ectU* and *ybdG* genes were lower than that of genes located closer to the promoter (Fig. 2C), probably reflecting the position of the *ectU* and *ybdG* mRNA segments close to the 3'-end of the poly-cistronic transcript. The reduced induction levels might simply be the consequence of a stability of the 3'-segment of the long mRNA. The transcript levels of *ectA*, *ectD*, *ectX* and *ectI* from *H. neptunium* cells grown with 0.5 M NaCl were 5- to 9.5-fold induced compared to cells grown in the absence of additional NaCl (Fig. 2F).

Overall, our transcriptional analysis revealed an osmotic up-regulation of the poly-cistronic *ect* gene clusters of *H. neptunium* and *Novosphingobium* sp. LH128, as would be expected for the function of ectoines as osmostress protectants (Pastor *et al.*, 2010, Czech *et al.*, 2018a, Hermann *et al.*, 2020). In addition, we also discovered that the genes for the various transport systems show approximately the same expression levels as the ectoine biosynthetic genes. These findings thus indicate that *H.*

neptunium and *Novosphingobium* sp. LH128 probably tightly coordinate the synthesis, import and export of ectoines under steady state osmotic stress conditions.

EctI and EctU are ectoine/hydroxyectoine importers but differ in their substrate profile

Our bioinformatic analysis predicts that EctI and EctU serve as importers for ectoines. To experimentally test this hypothesis, we performed osmostress protection assays (Haardt *et al.*, 1995). Hence, we introduced the *ectI* gene from *H. neptunium* and the *ectU* gene from *Novosphingobium* sp. LH128 into an *E. coli* strain (MKH13) lacking importers for osmostress protectants (Haardt *et al.*, 1995), including the ProP and ProU systems that are used for ectoine/hydroxyectoine import (Jebbar *et al.*, 1992, MacMillan *et al.*, 1999). Hence, this strain can be used for the functional analysis of heterologous compatible solute import systems (Kempf & Bremer, 1995). For these experiments, we used low-copy-number plasmids in which the *ectI* or the *ectU* genes were constitutively expressed from a *lac* promoter. The *E. coli* strain MKH13 carrying either plasmid pARO12 (*ectI*) or plasmid pANDA1 (*ectU*) was grown in a minimal medium (MMA) that contained a growth-limiting concentration of NaCl (0.8 M NaCl) (Haardt *et al.*, 1995). We added to these cultures 1 mM of 13 known osmostress protectants (Hoffmann & Bremer, 2017) and then monitored the growth yields of the cultures after a defined time (Fig. 4A,B). Both EctI and EctU proved to be ectoine and hydroxyectoine uptake systems; however, their substrate profile differed. The SSS-type transporter EctI from *H. neptunium* imported the following compatible solutes in addition to ectoine and hydroxyectoine: glycine betaine, homobetaine, proline betaine, and dimethylsulfoniopropionate (DMSP) (Fig. 4A). In contrast, the MFS-type transporter from *Novosphingobium* sp. LH128 exhibited a strict substrate specificity for ectoines (Fig. 4B).

In order to determine the apparent kinetic parameters for EctI and EctU for ectoine and hydroxyectoine, the *E. coli* MKH13[pARO12] (EctI) and MKH13[pANDA1] (EctU) strains were cultivated in 48-well mico-titer plates in MMA containing 0.8 M NaCl and increasing concentration of the compatible solutes ectoine, hydroxyectoine, or glycine betaine. The determination of the growth rates at different compatible solute concentrations allowed the fitting of the data according to the Monod equation (Monod, 1949), an equation describing the growth of cultures, which is based on the Michaelis-Menten equation. This revealed the maximum specific growth rate (µmax [h^{-1}]) and the substrate concentration that supports the half-maximal growth rate K_S [mM compatible solute] of the two studied importers. We point out that K_s values (Monod, 1949) are not identical with K_m-values of transporters but they allow an estimation of the corresponding substrate affinity of the transport system under study. EctU possesses a K_S of 18 ± 3 µM and 17 ± 3 µM for ectoine and hydroxyectoine, respectively (Fig. 5A, B), while the K_S values of EctI for these ectoines are 16 ± 3 µM and and 25 ± 3 µM (Fig. 5C, D). Judging from these values, both EctI and EctU are high affinity transporters for ectoines

and resemble those for ectoine uptake via the EctT protein from *Virgibacillus panthotenticus* (K_m: 44 ± 8 µM) (Kuhlmann *et al.*, 2011), and the EctP and ProP systems from *Corynebacterium glutamicum* (K_m: 63 ± 5 µM and 132 µM, respectively) (Peter *et al.*, 1998). We also measured the K_S value of EctI for glycine betaine and determined a value of 83 ± 7 µM (Fig. 5E). Consequently, EctI posesses affinities for the three tested substrates that are in a similar range and that should be well suited to scavange the osmostress protectants ectoine, hydroxyectoine and glycine betaine from environmental sources (Mosier *et al.*, 2013, Bouskill *et al.*, 2016, Warren, 2022).

Modelling of the overall fold of the EctI and EctU transporters and substrate docking of ectoines

To get a deeper insight into the newly indentified ectoine uptake systems EctI and EctU, we performed *in silico* modelling and substrate docking experiments. Both transport proteins revealed structural folds of previously characterized import systems. The SSS-type transporter EctI was found to possess a similar fold as the SiaT transporter from *Proteus mirabilis* (Fig. 6A,C) (PDB ID: 5NV9) (Wahlgren *et al.*, 2018). Docking of ectoine and hydroxyectoine into the EctI model indicated a potential substrate binding pocket rich in aromatic amino acids, that might to be suitable for the coordination of the two compatible solute molecules (Hanekop *et al.*, 2007, Kuhlmann *et al.*, 2008, Lecher *et al.*, 2009) (Fig 6B). Overlay of the structurally characterized SiaT transporter in complex with its substrate N-acetylneuraminic acid (Wahlgren *et al.*, 2018) with the EctI model in complex with ectoines shows that the position of the binding pockets are superimposable (RMSD 1.27Å) (Fig. 6D).

The structural model of MFS-type importer EctU reveals high similarity to the XylE xylose uptake system from *E. coli* (Fig. 7A,C) (PDB ID: 4GBY) (Sun *et al.*, 2012). Docking experiments using the EctU substrates ectoine and hydroxyectoine indicated a potential substrate binding pocket that, like substrate binding proteins for ectoines (Lecher *et al.*, 2009, Kuhlmann *et al.*, 2008, Hanekop *et al.*, 2007), possess several aromatic residues (Fig. 7B). Structural comparison of XylE bound to D-xylose and EctU in complex with ectoines highlights that the predicted substrate binding site of EctU is in close proximity to the experimentally determined xylose binding site found in XylE (Fig. 7D) (Sun *et al.*, 2012). Abbreviated alignments of different SSS-type and MFS-type import systems related to the EctI and EctU transporters for ectoines (Fig. 4A,B) are documented in Supplementary Fig. 7 and 8, respectively.

The EctX transporter protein enodes a putative export system for ectoines

Our bioinformatic analysis predicts that the EctX proteins from both *Novosphingobium* sp. LH128 and *H. neptunium* (Fig. 2A,D) could potentailly function as exporters for ectoines (see above). Although other interpretations are certainly possible, consistent with this prediction, we found that EctX was unable to confer osmostress protection to *E. coli* MKH13 cells when the same osmostress protection

assay was used that successfully identified EctI and EctU as import systems for compatible solutes (Fig. 4C). To functional verify the predicted role of EctX as export system, we used an *in vivo* assay system that has previously been established to assess the role of the multi-drug resistance exporter EmrE in glycine betaine efflux in *E. coli* (Bay & Turner, 2012). The assay is based on the idea, that *E. coli* wild-type cells will not be able to maintain an osmostress protective pool of imported compatible solutes when a presumed compatible solute exporter gene is expressed. *E. coli* can amass a broad range of osmostress protectants through the ProU ABC-transporter and through ProP, a member of the MFS family (Haardt *et al.*, 1995, MacMillan *et al.*, 1999).

We expressed the *Novosphingobium* sp. LH128 *ectX* gene under the control of an IPTG-inducible promoter on a plasmid in the *E. coli* wild-type strain MC4100 ($ProP^+$ $ProU^+$). We propagated these cells under otherwise growth-restricting osmotic conditions (MMA with 0.8 M NaCl) in the presence of various osmostress protectants. In the absence of compatible solutes, growth of strain MC4100 carrying either the cloning vector pTrc99 or the *ectX*$^+$ plasmid pLC108 was severely restricted. However, when compatible solutes were present, both strains grew well as a result of the activities of the ProU and ProP import systems (Fig. 8A). However, among the 13 tested compatible solutes, three compounds (ectoine, hydroxyectoine, L-proline) were unable to provide adequate osmostress protection in the strain expressing *ectX,* while the control strain harboring the empty vector was not affected in its growth at high salinity in the presence of these three osmostress protectants (Fig. 8A). Hence, synthesis of EctX specifically restricts the growth of those *E. coli* cells that rely for their osmostress protection on the accumulation of ectoines, and to a lesser degree also by L-proline. These data are consistent with the hypothesis that EctX serves an exporter, and our *in vivo* data demonstrates that it possesses a defined, yet restricted, substrate profile that primarily comprises ectoine and hydroxyectoine.

We analyzed the phenotype conferred by the expression of *ectX*$^+$ onto osmotically stressed *E. coli* cells further by growing the MC4100(pTrc99) and MC4100(pLC108) strains in the presence of compatible solutes. Growth of strain MC4100 ($ProU^+$ $ProP^+$) carrying the empty vector pTrc99 occurred unimpeded in high salinity media (MMA with 0.8 M NaCl) when the compatible solutes glycine betaine, proline betaine, L-proline, ectoine, or hydroxyectoine were provided at a final concentration of 1 mM (Fig. 8B). In contrast, growth of the *E. coli* cells expressing the *ectX*$^+$ gene was prevented when 1 mM ectoine, hydroxyectoine, or L-proline was present in the medium, while the cells that were provided with the same concentration of glycine betaine or proline betaine were able to commence growth (Fig. 8C). This effect is dependent on the inducer IPTG as shown in a growth experiment with hydroxyectoine (Fig. 8D, E), as the *ectX*$^+$ gene is expressed from the *lac* promoter carried by the pTrc99 plasmid (Amann *et al.*, 1988).

We also aimed to analyze EctX via modelling and docking approaches. Modelling of EctX revealed a structural similarity to the multidrug exporter MdfA from *E. coli* (PDB ID: 4ZOW) (Heng *et al.*, 2015) with an RMSD value of 3.95 Å (Supplementary Fig. 9). An abbreviated protein sequence alignment of EctX-related putative MFS-type exporters found in the genetic context of *ect* gene clusters are represented in Supplementary Fig. 10. However, attempts to dock the ectoine/hydroxyectoine molecules into the predicted EctX structure did not reveal a clear binding site for these compounds.

The ybdG gene from Novosphingobium sp. LH128 likely encodes a functional mechanosensitive channel

Mechanosensitive channels are safety valves whose transient gating protect cells against rupture when water rapidly enters the cell in response to a sudden and severe osmotic down-shock (Booth, 2014, Cox *et al.*, 2018). In 23 representatives of *ect* gene clusters we found genes that potentially could encode proteins belonging to the MscS-family of mechanosensitive channels (Booth & Blount, 2012). These differ in the length and membrane topology. The presumptive mechanosensitive channel protein from *Novosphingobium* sp. LH128 is related to the YbdG protein (405 amino acids) from *E. coli* (54% amino acid sequence identity) and comprises 415 amino acids (Amemiya *et al.*, 2019, Edwards *et al.*, 2012). It only shares only 23% amino acid sequence identity to the *E. coli* MscS protein (286 amino acids). The two YbdG proteins are predicted to contain five transmembrane segments with a core that resembles in its topology that of the functionally and structurally characterized *E. coli* MscS-type protein which exhibiting three trans-membrane segments (Reddy *et al.*, 2019, Zhang *et al.*, 2021, Hu *et al.*, 2021) (Fig. 9A,B,C).

Previous studies identified YbdG as a threshold-setting mechanosensitive channel playing a minor role in protecting osmotically down-shocked cells from lysis (Schumann *et al.*, 2010, Edwards *et al.*, 2012). In a more recent study, YbdG was described to function as a component of a mechano-sensing system transmitting signals generated by external osmotic changes to yet ill-defined intracellular factors (Amemiya *et al.*, 2019). Typically, the functionality of a given Msc channel can be assessed by a complementation assay in an *E. coli s*train lacking all MscL-, MscS-, and MscM-type channels, as this mutant (MJF641; referred to as strain Δ7) is highly sensitive against severe osmotic down-shocks (Edwards *et al.*, 2012). We expressed a codon-optimized version of the *Novosphingobium* sp. LH128 *ybdG* gene (Fig. 2A) under the control of an IPTG-inducible promoter in the MJF641 (Δ7 mutant) indicator strain and assessed cell viability under suddenly imposed osmotic down-shift conditions (Levina *et al.*, 1999). For this experiment, strain MJF641 (Δ7) carrying the *Novosphingobium* sp. LH128 *ybdG* gene on the expression plasmid pLC19 was grown in MMA with 0.3 M additional NaCl and expression of the *ybdG* gene was induced by adding IPTG to the growth medium. The cells were

then confronted with a rapid osmotic downshift by exposing them to a growth medium containing no additional amounts of NaCl. The parent (strain FRAG1) of the Δ7 mutant-strain carrying the empty cloning vector pTrc99a survived this osmotic downshift with a recovery rate of nearly 100% (99.5% ±1.6%). In contrast, only 4.3% ± 0.7% of the cells of the Δ7 mutant-strain MJF641 (pTrc99a) survived this treatment (Fig. 9D) as expected from previously reported data (Edwards *et al.*, 2012, Levina *et al.*, 1999). When MJF641 (pLC19; *ybdG*$^{+}$) was subjected to such an osmotic down-shock regiment, the *Novosphingobium* sp. LH128 YbdG protein rescued cellular survival to a large extent (60.3% ± 8.6% surviving cells) (Fig. 9D).

Discussion

In comparison with the energy-demanding synthesis of ectoines (Oren, 1999), import of these stress protectants provides microbial cells with resource-preserving means to counteract the detrimental effects of high osmolarity on cellular physiology and growth (Pastor *et al.*, 2010, Kunte *et al.*, 2014, Czech *et al.*, 2018a, Hermann *et al.*, 2020). Most of the previously functionally characterized importers for ectoine and hydroxyectoine are stand-alone systems. These can be found both in microorganisms producing ectoines and in those lacking this biosynthetic trait. So far, four different types of bacterial ectoine/hydroxyectoine importers were known and comprise both multi-component substrate-binding-protein dependent systems (ABC, TRAP-T), and single component transporters (BCCT, MFS). These differ in their mode of energization, as transporters are represented that rely for their activity either on the hydrolysis of ATP, or on proton or sodium trans-membrane gradients. We have now identified a new type of ectoine/hydroxyectoine transport system (EctI) (Fig. 4A) that belongs to the sodium-solute-symporter family (SSS), transporters that import Na^{+} ions along with their specific substrate(s) into the cell (Henriquez *et al.*, 2021).

Through a comprehensive bioinformatic analysis of 1,103 ectoine/hydroxyectoine biosynthetic gene clusters, we found that about 37% of them possess transporter genes in the corresponding genomic context (Table 1). Amino acid sequence alignments, *in vivo* studies, *in silico* modelling, and structural comparisons support our proposal that the transporters encoded by these genes are in all likelihood importers for ectoines. Obviously, further functional studies are required to confirm or refute this hypothesis. In addition, structural and biochemical analysis will be required to identify the true architecture of the substrate binding site in the various types of the presumed ectoine/hydroxyectoine transporters, and physiological studies are needed to reveal their complete substrate profile. It is already known that transporters for ectoines can possess either narrow or broader substrate specificity (Kuhlmann *et al.*, 2011, Du *et al.*, 2011, MacMillan *et al.*, 1999, Vermeulen & Kunte, 2004, Peter *et al.*, 1998, Gregory & Boyd, 2021). In our analysis of the ectoine/hydroxyectoine synthesis and transporter gene clusters from the soil bacterium *Novosphingobium* sp. LH128 and the

marine bacterium *H. neptunium* (Fig. 2A,D) we found examples for both transporter characteristics. EctU is specific for ectoines, while EctI possess transport activity not only for ectoines but also for four additional compatible solutes (Fig. 4A,B).

Our bioinformatic overview of ectoine/hydroxyectoine biosynthetic gene clusters indicate that in many of the corresponding microorganisms, synthesis and uptake of these stress protectants are probably genetically and physiologically interconnected. Indeed, we proved this directly through the transcriptional analysis of the osmotically induced poly-cistronic ectoine/hydroxyectoine biosynthetic and transporter operons from *Novosphingobium* sp. LH128 and *H. neptunium* (Fig. 2 C,F). Genetic co-regulation of these two osmostress relieving traits makes physiologically sense, as it allows the osmotically challenged cell to concomitantly adjust its compatible solute pool to the prevalent osmolarity in its surroundings through two different mechanisms.

Besides their obvious role in the high-affinity acquisition of environmental osmostress protectants, transporters for these compounds have also been implicated to function as recycling systems for newly synthesized compatible solutes extruded by the producer cells. This role becomes apparent in mutants lacking transporters for the type of the dominant compatible solute synthesized by a given bacterial cell (Grammann *et al.*, 2002, Hoffmann *et al.*, 2012, Lamark *et al.*, 1992, Mikkat & Hagemann, 2000, Nyyssölä & Leisola, 2001, Schubert *et al.*, 2007, Börngen *et al.*, 2010). Hence, microorganisms seem to engage in a synthesis – release – recovery cycle, a process that might be physiologically connected to the fine-tuning of turgor as the cell doubles its volume prior to division (Misra *et al.*, 2013), or when osmotic down-shifts are either moderate, or occur slowly (Hoffmann *et al.*, 2012, Börngen *et al.*, 2010). A variation of this theme has been addressed as the “pump and leak” mechanism where a given compatible solute is imported through an osmotically stimulated transporter and subsequently partially leaks (or is actively extruded) from the cell, apparently in an effort to precisely adjust the magnitude of turgor to the prevalent cellular and environmental circumstances (Börngen *et al.*, 2010).

We found 23 ectoine/hydroxyectoine biosynthetic gene clusters that contain a gene encoding a member of the MscS-family of mechanosensitive channels (Booth & Blount, 2012). Judging by the degree of homology, amino acid sequence length and predicted number of trans-membrane segments, these proteins seem to belong to different sub-groups of the wider MscS family. In contrast to the high degree of evolutionary conservation of MscL-type channels (Booth & Blount, 2012), MscS-type mechanosensitive channels present in both *Pro-* and *Eukarya* are remarkable diverse with respect to their structure (Wilson *et al.*, 2013, Deng *et al.*, 2020, Cox *et al.*, 2018). The Msc protein from *Novosphingobium* sp. LH128 that we have physiologically characterized (Fig. 2A) is related to the YbdG system from *E. coli* (Edwards *et al.*, 2012, Schumann *et al.*, 2010, Amemiya *et al.*, 2019), as evidenced by amino acid sequence comparison and modelling approaches (Fig. 9B,C). The physiological role of

the *E. coli* YbdG protein is debated in the literature. It was either described as a bona-fide mechanosensitive channel active in the canonical osmotic downshift assays (Levina *et al.*, 1999); however, it was not active in electrophysiological patch-clamp studies unless a gating mutation (V229/A) was introduced (Schumann *et al.*, 2010). Alternatively, the *E. coli* YbdG protein was proposed to function primarily as a component of a mechanosensory system for cellular adaptation to high osmolarity souroundings (Amemiya *et al.*, 2019). However, this conclusion rests on the analysis of a gain of function mutation (I167/T) causing cellular effects different from those observed for the wild-type YbdG protein (Amemiya *et al.*, 2019).

We provide evidence for the functionality of the *Novosphingobium* sp. LH128 YbdG protein as a mechanosensitive channel through a well-established complementation assay (Fig. 8C) (Levina *et al.*, 1999) using an *E. coli* mutant lacking all known mechanosensitive channels (Edwards *et al.*, 2012). As the *ect* biosynthetic and *ybdG* genes in *Novosphingobium* sp. LH128 are co-transcribed under persistent osmotic stress conditions (Fig. 2A,B,C), it seems that these bacteria already prepare themselves at continued high osmolarity for an inevitably coming osmotic down-shock. None of the few *ect* gene clusters present in archaea that we have analyzed contained a transporter gene but some of them encode a member of the MscS-family different from YbdG, as already reported and experimentally verified for the archaeon *Nitrosopumilus maritimus* (Widderich *et al.*, 2016).

In *C. glutamicum,* an MscS-type protein (MscCG) was specialized in the course of evolution to function not only as a mechanosensitive channel but also as an export system for L-glutamate (Börngen *et al.*, 2010, Kawasaki & Martinac, 2020, Becker *et al.*, 2013). This particular representative of MscS-type channels raises the question if any of the presumptive Msc proteins encoded in *ect* biosynthetic gene clusters possess specificity for ectoines. The crystal structure of YbdG-type channels is not known. Lipids play an important role for the gating mechanism of MscL- and MscS-type channels (Pliotas *et al.*, 2015, Kapsalis *et al.*, 2019). Hence, structural, biochemical and functional analysis of YbdG-type proteins should be rewarding to further our understanding of the structure/function relationship of this group of channel-forming proteins, and the mechanism underlying their gating behavior (Amemiya *et al.*, 2019, Schumann *et al.*, 2010).

Release of ectoines under steady-state high osmolarity growth conditions has been observed for both natural ectoine producers and for synthetic microbial cell factories expressing heterologous ectoine/hydroxyectoine biosynthetic genes (Schubert *et al.*, 2007, Eilert *et al.*, 2013, Czech *et al.*, 2018b, Czech *et al.*, 2016, Jebbar *et al.*, 1992, Vandrich *et al.*, 2020). This process occurs independent of mechanosensitive channels (Vandrich *et al.*, 2020, Czech *et al.*, 2016, Czech *et al.*, 2019b), raising the possibility that the release of ectoines under these conditions is mediated either by substrate specific excretion systems, or excretion systems possessing a broad substrate profile. Indeed, kinetic

evidence for the existence of a glycine betaine release system different from those of mechanosensitive channels has been provided for *Lactobacillus plantarum* (Glaasker *et al.*, 1996).

Dedicated excretion systems for various microbial metabolites (e.g., amino acids, sugars, antibiotics, signaling molecules) exist widely in microorganisms (Fritts *et al.*, 2021, Jones *et al.*, 2015, Bay & Turner, 2012, Eggeling & Sahm, 2003, Perez-Garcia & Wendisch, 2018). We see *a priori* no reason that such systems would also not exist in bacteria for compatible solutes. Indeed, we discovered a gene (*ectX*) located in 56 out of 1,103 ectoine/hydroxyectoine biosynthetic gene clusters whose encoded protein is related to various structurally and functionally characterized multi-drug resistance efflux systems; e.g. YajR, MdfA, MdtM and YcaD (Law & Alegre, 2018, Jiang *et al.*, 2013, Heng *et al.*, 2015, Pade & Hagemann, 2014). Notably, the *ycaD* gene is co-expressed with the biosynthetic genes for the compatible solute glucosylglycerol in *Stenotrophomonas rhizophila*, a microorganism that secrets this osmolyte in stationary phase cultures (Roder *et al.*, 2005, Alavi *et al.*, 2013). EctX and YcaD share a sequence identity of 33%.

What is the physiological evidence that EctX is an excretion system for ectoines? It is expected that cells overexpressing an excretion system for a given compatible solutes will be unable to maintain an adequate osmostress protective compatible solute pool (Bay & Turner, 2012). The substrate profile of a putative export system for compatible solutes will thus be revealed *in vivo* when some of the tested exogenously provided compatible solutes will no longer afford osmostress protection when a putative exporter-encoding gene is over-expressed. Exactly this phenotype was observed when the *Novoshingobium* sp. LH128 *ectX* gene was expressed from a recombinant plasmid in *E. coli* cells grown at high salinity in the presence of various osmostress protectants. From 13 tested osmostress protectants, EctX production selectively strongly inhibited growth of ectoine and hydroxyectoine exposed cells, and also, but to a limited degree, that of cells exposed to the compatible solute L-proline (Fig. 7).

Overall, the *guilty by association* approach (Kumar *et al.*, 2014, Zhao *et al.*, 2013) that we have taken in our study proved to be rewarding on several levels, including the identification of a new type of ectoine/hydroxyectoine importer (EctI) and a system for the extrusion (EctX) of these types of stress protectants. At the same time, important new questions are raised with respect to balancing the activities of the functionally antagonistic importer and exporter transport systems under steady-state high osmolarity conditions in those microorganisms harboring corresponding genes expressed as part of the same osmotically induced operon (Fig. 2). Co-expression of ectoine/hydroxyectoine biosynthetic genes with genes encoding mechanosensitive channels under high osmolarity growth conditions seem less problematic as the opening of mechanosensitive channels requires a “force from lipids” stimulus that is generated during substantial osmotic downshifts (Cox *et al.*, 2018, Booth, 2014, Levina *et al.*, 1999, Pliotas *et al.*, 2015, Wilson *et al.*, 2013).

From an ecological perspective, most microorganisms do not live as solitary entities but are rather part of complex multi-species microbial communities or biofilms (Flemming & Wuertz, 2019). Interestingly, the ectoine biosynthetic operon and the associated EctU importer, EctX exporter, and YbdG-type mechanosensitive channel genes (Fig. 2A) are among the most highly induced genes upon imposition of osmotic shock and chronic stress within biofilms formed by the soil bacterium *Novosphingobium* sp. LH128 (Fida *et al.*, 2012). Biofilms are complex assemblies of microorganisms encased in a self-produced matrix and are shaped and strained by osmotic forces (Rubinstein *et al.*, 2012, Yan *et al.*, 2017, Bremer & Krämer, 2019). Cells encased in biofilms profit from the exchange of nutrients (Diaz-Pascual *et al.*, 2021, Fritts *et al.*, 2021) and stress protectants. For instance, the compatible solute glycine betaine serves as a public good in assemblages of the marine bacterium *Vibrio cholerae* (Kapfhammer *et al.*, 2005). Supported by exporters for ectoines, we envision a similar function for these compatible solutes in biofilms, or in complex microbial communities.

Importers and exporters of compatible solutes and mechanosensitive channels are important elements of the overall adaptation network balancing the cell's response to fluctuating osmotic circumstances in their ecophysiologically varied niches (Wood, 2011, Booth, 2014, Bremer & Krämer, 2019). Our description of the molecular identity of the first ectoine/hydroxyectoine specific export system (EctX) should aid our understanding of the behavior of osmotically stressed individual microbial cells and communities. In addition, EctX and other types of export systems should also support further strain development of microbial cell factories (Borodina, 2019, Jones *et al.*, 2015, Perez-Garcia & Wendisch, 2018) for the production of ectoines, commercially valuable extremolytes and chemical chaperones (Becker & Wittmann, 2020, Kunte *et al.*, 2014, Pastor *et al.*, 2010, Liu *et al.*, 2021). Hence, the synthetic implementation of an EctX-type export system in an ectoine/hydroxyectoine producing cell might result in a hypersecreting production host.

Experimental procedures

Chemicals

Ectoine and hydroxyectoine were kindly provided by the bitop AG (Witten, Germany). Acetonitrile (HPLC-grade) was obtained from VWR International GmbH (Darmstadt, Germany). Chloramphenicol and ampicillin, and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), Serva Electrophoresis GmbH (Heidelberg, Germany) and Carl Roth GmbH (Karlsruhe, Germany). Enzymes for DNA manipulations were obtained from Thermo Fisher Scientific GmbH (Dreieich, Germany), Roche Diagnostics GmbH (Mannheim, Germany) and New England BioLabs GmbH (Frankfurt, Germany).

Bacterial strains, media and growth conditions

The *E. coli* K-12 wild type laboratory strain MC4100 is the parent of the strain MKH13 [Δ(*proP*)2 Δ(*proU::spc*)608] (Spc^r)] carrying defects in the genes encoding the ProP or ProU compatible solute uptake systems. It is also defective in the BetT choline importer and the genes encoding the enzyme for glycine betaine synthesis from choline (Haardt *et al.*, 1995) (Supplementary Table S1). The *E. coli* strain MJF641 is a derivative of the wild-type strain FRAG1 and lacks all currently known *mscL*, *mscS, mscK* and *mscM*-type mechanosensitive channel genes (the strain is also referred to as Δ7) (Edwards *et al.*, 2012). These strains were kindly provided by I.R. Booth (University of Aberdeen, United Kingdom). All *E. coli* strains were routinely maintained on Luria Bertani (LB) agar plates and propagated in liquid LB medium (Miller, 1972). When they contained a recombinant plasmid, ampicillin (100 µg ml^{-1}) or chloramphenicol (30 µg ml^{-1}) was added to the growth medium, as required by the antibiotic resistance determinant carried by the particular plasmid (Supplementary Table S2). For the functional assessment of genes encoding importers or exporters for ectoines, the *E. coli* strains were grown in minimal medium A (MMA) supplemented with 0.2% (w/v) glucose as the carbon source, 1 mM $MgSO_4$, and 1.5 µM (= 0.5 mg l^{-1}) thiamine (Miller, 1972). The osmolarity of the growth medium was adjusted by adding various concentrations of NaCl, as specified in the individual experiments. Shake-flask cultures were incubated at 37 °C in a shaking water bath set to 220 rpm. The osmolarity of media was measured according to the manufacturer's protocol using a Gonotec freezing point osmometer (Osmomat 3000basic, Gonotec, Berlin, Germany).

Osmostress protection assays with *E. coli* strains (MC4100 or MKH13) (Haardt *et al.*, 1995) carrying different plasmids were conducted in 100-ml Erlenmeyer flasks (culture volume of 20 ml) by growing the cells (at 37° C) in MMA containing 0.8 M NaCl in absence or presence of the tested compatible solutes (1 mM final concentration). The cultures were inoculated to an OD_{578} of 0.1 and their growth yield was recorded after 24, 30 and 48 h by measuring their OD_{578}. In various cases, such as for the determination of the apparent k_m (in terms of the K_s based on the Monod equation) (Monod, 1949) of the EctI and EctU transporters, cultures were grown in 48-well plates. Each well contained 500 µl of medium and the well plate was incubated in an Epoch 2 microplate spectrophotometer (Biotek, Bad Friedrichshall, Germany) at 37° C with double orbital vigorous shaking. The growth of the cultured was monitored every hour for 48 h or 96 h, respectively.

The *Novosphingobium* sp. LH128 strain (Bastiaens *et al.*, 2000) was kindly provided by Dirk Springael (KU Leuven, Belgium) and cultivated in LB or Spizizen's minimal medium (SMM), with 0.5% (wt/vol) glucose as the carbon source. The medium was supplemented with L-tryptophan (20 mg liter^{-1}), L-phenylalanine (18 mg liter^{-1}), and a trace element solution (Harwood & Archibald, 1990). This medium possesses a measured osmolarity of 377 mOsm kg^{-1}. Cells were grown aerobically at 30° C in 100-ml baffled Erlenmeyer flasks with a culture volume of 10 ml in a shaking water bath set at 220

rpm; the medium was inoculated to OD_{578} 0.1 from a pre-culture. The growth of the bacterial cultures was monitored spectrophotometrically at a wavelength of 578 nm (OD_{578}). The salinity of the growth medium was raised by adding appropriate volumes of NaCl solutions from a 5 M stock solution prepared in H_2O.

The *H. neptunium* strain LE670 (ATCC 15444) (Badger *et al.*, 2005) was a kind gift from Martin Thanbichler (Philipps University Marburg, Germany) and was cultivated aerobically in Difco marine broth 2216 (MB) (BD Biosciences, Heidelberg, Germany) at 30° C under vigorous shaking (220 rpm) in baffled flasks. This medium possesses a measured osmolarity of 924 mOsm kg^{-1}. For the cultivation at increasing salinities, the MB medium was prepared at a 2-fold concentration and then diluted to 1-fold MB with distilled water, and if needed, appropriate volumes of NaCl from a 5 M stock solution were added.

Recombinant DNA procedure and construction of plasmids

Routine manipulations of plasmid DNA, the construction of recombinant plasmids and the isolation of chromosomal DNA from *H. neptunium* and *Novosphingobium* sp. LH128 were carried out using standard techniques. The genes *ectI* (*H. neptunium*) and *ectU* (*Novosphingobium* sp. LH128) were amplified from chromosomal DNA using the synthetic primers Hypho5_EcoRI_for and Hypho3+5_HindIII_rev (for *ectI*) and EctU EcoRI for and EctU HindIII rev (for *ectU*) (Supplementary Table S3). The obtained DNA fragments contained artificial EcoRI and HindIII restriction sites at their 5'-ends and were cloned into the low-copy-number plasmid pHSG575 (Cm^r) (Takeshita *et al.*, 1987) that had been cut with EcoRI and HindIII. This yielded the plasmids pARO12 (*ectI* from *H. neptunium*) and pANDA1 (*ectU* from *Novosphingobium*), respectively.

For the heterologous expression of the *mscS*-type *ybdG* and *ectX* genes from *Novosphingobium* sp. LH128 in *E. coli*, the genes were cloned into the expression vector pTrc99a (Amann *et al.*, 1988). In case of *ybdG*, a codon-optimized synthetic *ybdG* gene (NCBI Genbank Accession number: MK761064.1; https://www.ncbi.nlm.nih.gov/nuccore/MK761064.1) was amplified from the plasmid obtained from the supplier (GeneArt Gene Synthesis, Thermo Fisher Scientific, Dreieich, Germany) by PCR using the custom synthesized DNA primers MscS_Sphingo EcoRI for and MscS_Sphingo HindIII rev (Supplementary Table S3). The (*Ns*)*ectX* gene was amplified using the EctX_pTrc99a NcoI for and EctX_pTrc99a BamHI rev DNA primers. The resulting DNA fragments carried NcoI and HindIII restriction sites, respectively; appropriate DNA-sequences were attached either to the 5'or to the 3'prime ends of the PCR product, thereby enabling its directional insertion into the expression vector pTrc99a (Amann *et al.*, 1988). This positioned the transcription of the *ybdG* gene under the control of the LacI/IPTG controlled *lac* promoter carried by the pTrc99a vector and resulted in the isolation of plasmid pLC19. Construction of plasmid pLC108 harboring the *ectX* gene from

Novosphingobium sp. LH128 under the control of the *lac* promoter present on pTrc99a were constructed in an analogous fashion, except that the gene was amplified from *Novosphingobium* sp. LH128 chromosomal DNA. All plasmids used in this study are listed in (Supplementary Table S2).

Isolation of total RNA and transcription analysis of the ectoine/hydroxyectoine biosynthetic gene cluster

Total RNA from *H. neptunium* and *Novosphingobium* cells cultured under different salinity conditions was isolated as reported previously (Fida *et al.*, 2012). In brief, *H. neptunium* and *Novosphingobium* sp. LH128 were cultivated at 30° C in MB or SMM, respectively, with or without the addition of NaCl until the cultures reached an OD_{578} of about 1 was reached. 1-ml of cell culture was mixed with 2 ml of fresh RNA protect bacterial reagent (QIAGEN, Hilden, Germany), vortexed for 5 sec, incubated at room temperature for 5 min, and the cells were then harvested at 2,415 x g for 10 min (at room temperature). Total RNA was immediately extracted using Promega SV total RNA extraction kit (Promega, Madison, USA) with minor modifications of the protocol (centrifugation was performed at 15,115 x g instead of at 14,000 x g and 200 µl of Tris-EDTA containing 0.4 mg ml^{-1} lysozyme was used instead of 100 µl). The RNA was eluted with 60 µl of RNase-free water. Traces of remaining DNA were removed by using the Turbo DNA-free kit according to the manufacturer's protocol (Ambion, Invitrogen, Thermo Fisher Scientific, USA).

The isolated total RNA was used for one-step RT-PCR assays to assess the transcriptional organization of the *ect* operon from *H. neptunium* and *Novosphingobium* sp. LH128. To analyze whether the *ectR-ectABCD-ask_ect-ectX-ectU-ybdG* genes are co-transcribed, 11 (*Novosphingobium* sp. LH128) or eight (*H. neptunium*) intergenic regions of the putative *ectR-ectABCD-ask_ect-ectX-ectI* operon were amplified from the isolated total RNA using the Qiagen One Step RT-PCR Kit (QIAGEN, Hilden, Germany) and custom synthesized DNA primers (Eurofins MWG, Ebersberg, Germany) (Supplementary Table S3). As controls, DNA regions between genes that were divergently transcribed we also amplified (see Fig. 2A). To ensure that the formed PCR products did not result from DNA contaminations of the total RNA sample used for the RT-PCR reaction, a PCR reaction with the same primers was performed with total RNA as a PCR template. This resulted in no amplification of the potential DNA fragments.

Transcriptional analysis by quantitative RT-PCR

For studying the expression of the ectoine/hydroxyectoine biosynthesis gene cluster in *H. neptunium* and *Novosphingobium* sp. LH128 under different osmotic growth conditions, cells that had been cultured either at low (0 mM NaCl), or high salt (500 mM NaCl for *H. neptunium* and 600 mM NaCl for *Novosphingobium* sp. LH128). Total RNA was extracted from these cells as described above. The

absence of DNA contamination was ascertained by PCR analysis. The relative abundance of the *ectA, ectD, ectX, ectU* and *mscS* mRNA in salt-stressed compared to non-stressed cells was determined by real-time PCR in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, München, Germany) with the LightCycler RNA Master SYBR green I kit (Roche Diagnostics, Mannheim, Germany). Each reaction of the one-step RT-PCR was conducted in a 20-µl volume containing 50 ng template RNA, 0.5 µM of each primer, 3.25 mM $Mn(OAc)_2$ and 7.5 µl of LightCycler RNA Master SYBR green I. The PCR primer sets used are shown in (Supplementary Table S3). The size of the amplified PCR products range between 120 and 160 bp. The PCR cycling conditions were used as described in the manufacturer's instructions with 30 min of reverse transcription to cDNA at 61° C, followed by an initial denaturation step at 95° C for 2 min. Then 45 PCR cycles were run with denaturation at 95° C for 5 sec, annealing at 56° C for 10 sec and extension at 72° C for 10 sec. After this qRT-PCR, a melting curve for quality control was obtained: 95° C for 10 s, followed by a temperature ramp from 65 to 95° C in 0.5° C steps for 15 sec. The relative expression of each gene under the tested conditions was determined by using the *gyrB* transcript level in *H. neptunium* and *Novosphingobium* sp. LH128 cells as the standard. Induction values are expressed as the log 2 of the ratio of the value determined for the salt-stressed cells over the value determined for the non-stressed control. All of the experiments were conducted in at least two independent biological replicates, and relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Modelling and docking of three-dimensional protein structures and preparation of figures of crystal structures

Amino acid sequences of the studied proteins were retrieved from the IMG/M database (Chen *et al.*, 2021). Structural models of the EhuB-, TeaA-, OpuBC- and ProX-type substrate binding protein homologs, the BCCT-type transporter of *V. alginolyticus,* the EctI transporter from *H. neptunium* and the EctU and EctX transporter and mechanosensitive channel from *Novosphingobium* sp. LH128 were built using Alphafold 2 and SWISS Model (Jumper *et al.*, 2021, Waterhouse *et al.*, 2018). For ligand docking, the obtained models were manually inspected to avoid clashes of amino acid site chains. The ligands ectoine and hydroxyectoine, were extracted from the crystal structures 2VPN and 2Q89 deposited in the PDB database, respectively. These molecules were docked into the obtained homology models utilizing a combination of AutoDock as a docking engine and the DrugScore2018 distance-dependent pair-potentials as an objective function (Hanekop *et al.*, 2007, Kuhlmann *et al.*, 2008, Goodsell *et al.*, 1996, Sotriffer *et al.*, 2002, Dittrich *et al.*, 2019). During docking, default parameters were used, except for the clustering RMSD cutoff, which was set to 2.0 Å. For the transport proteins the grid size was 45 Å around ectoine to include the complete transport proteins. The possible binding sites for the ligands under study were compared to the known binding sites of ectoine and

hydroxyectoine previously obtained by structural analysis of the ectoine/hydroxyectoine EhuB substrate binding (Hanekop *et al.*, 2007). Based upon these data, two potential positions for the ligands were selected and redocked into the transporter model resulting in one defined location of the ligand. This procedure was independently performed for the EctU and EctI proteins. Structures of all proteins were visualized and analyzed using the PyMol Molecular Graphics System suit (https:// www.pymol.org) (Delano, 2002).

HPLC analysis of ectoine and hydroxyectoine content

Low molecular weight organic solutes were extracted from cell pellets of *Novosphingobium* sp. LH128 and *H. neptunium* with 20% ethanol. For this purpose, the cell pellets were resuspended in 1-ml 20% ethanol and were shaken for 1 h at room temperature. After centrifugation at 16,000 x g (4° C, 30 min) to remove cell debris, the ethanolic extracts were transferred into fresh Eppendorf tubes, and the ethanol was removed by evaporation (at 65° C for 20 h). The resulting dried material was suspended in 100 µl of distilled water and insoluble material was removed by centrifugation (16,000 x g at 4° C for 30 min). The extracted samples and the cell-free culture supernatants were diluted ten-fold with distilled water and acetonitrile (the end concentration of acetonitrile was 50%) and analyzed for their ectoine/hydroxyectoine content by isocratic high-performance liquid chromatography (HPLC) (Kuhlmann & Bremer, 2002). For these measurements an Agilent 1260 Infinity LC system (Agilent, Waldbronn, Germany) and a GROM-SIL Amino 1PR column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was employed as described (Kuhlmann & Bremer, 2002) with the exception that a 1260 Infinity Diode Array Detector (DAD) (Agilent) was used instead of the previously used UV/Vis detector system. The ectoine content of samples was quantified using the OpenLAB software suite (Agilent). Standard curves for the calculation of the ectoine and hydroxyectoine concentrations were determined with commercially available samples (obtained from bitop AG, Witten, Germany).

Functional complementation of the mechanosensitive-channel-defective E. coli mutant MJF641 by the Novosphingobium sp. LH128 YbdG protein

For the analysis of cell viability of strains suddenly exposed to an osmotic down-shock (Levina *et al.*, 1999), the *E. coli* strains FRAG1(pTrc99a), MJF641(pTrc99a) and MJF641(pLC19) were cultured in the previously described chemically defined growth medium (Edwards *et al.*, 2012). It has a measured osmolarity of 235 mOsm kg^{-1}. For cells that were grown at high salinity, 0.3 M NaCl were added to the basal medium; this modified medium had a measured osmolarity of 730 mOsm kg^{-1}. The *E. coli* strains FRAG1 and MJF641 (Edwards *et al.*, 2012) harboring different plasmids were inoculated in 5 ml LB medium containing ampicillin (100 µg ml^{-1}) and were grown for 5 h. Cells were subsequently transferred into citrate-phosphate medium (Edwards *et al.*, 2012) and incubated over-night at 37° C.

Cells were then diluted to an OD_{578} of 0.05 into 20 ml minimal medium (Edwards *et al.*, 2012), or into 20 ml medium that contained 0.3 M NaCl; the cultures were subsequently grown to an OD_{578} of 0.15. At this point, IPTG was added to the cultures (final concentration 1 mM) to induce the activity of the LacI regulated *lac* promoter present on the back-bone of the expression plasmid pTrc99a (Amann *et al.*, 1988), thereby triggering the transcription of the codon-optimized *Novosphingobium* sp. LH128 *ybdG* gene present on plasmid pLC19 (NCBI Genbank Accession number: MK761064.1; https://www.ncbi.nlm.nih.gov/nuccore/MK761064.1). Growth of the cells was subsequently continued until they reached an OD_{578} of about 0.35. These cultures were then diluted 20-fold into pre-warmed medium (37° C) containing 1 mM IPTG with (no osmotic down-shock) or without (osmotic down-shock) 0.3 M NaCl; the cells were subsequently incubated at 37° C in a shaking water bath for 30 min. To determine the number of cells that survived the osmotic down-shock, 50-µl samples were taken after 30 min of incubation from these cultures, serial diluted in four independent sets in media with corresponding osmolarities and four 5 µl samples of the osmotically downshifted cells were then spotted onto Luria-Bertani (LB) agar plates (Miller, 1972). Those from the high osmolarity grown cells were spotted onto LB agar plates with a total NaCl content of 0.3 M NaCl. Colony-forming units were determined after overnight incubation of the LB plates at 37 °C.

Database searches for ectoine biosynthesis-related proteins

For the bioinformatical analysis for potential ectoine/hydroxyectoine transporter genes, we searched the IMG/M database (Chen *et al.*, 2021) for ectoine biosynthetic gene clusters (only fully sequenced genomes were analyzed) using the EctC protein from *P. lautus* (https://www.uniprot.org/uniprot/A0A2A5LBI6) (Czech *et al.*, 2019a) as a search query as reported previously (Mais et al. 2020). The bioinformatics tools available at the DOE Joint Genome Institute website (Chen *et al.*, 2021) were then used to manually analyze all putative ectoine gene clusters from the obtained dataset for the presence of transporter genes in their direct neighborhood. The organization of these genes was visualized using Adobe Illustrator. Protein sequences were aligned and compared using the MUSCLE alignment algorithm as implemented in SnapGene (https://www.ebi.ac.uk/Tools/msa/muscle/; https://www.snapgene.com).

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Supporting information

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

Fig. S1. Schematic illustrations of ectoine/hydroxyectoine gene clusters encoding putative transporters and mechanosensitive channels in their neighborhood.

Fig. S2. Modelling and protein sequence alignment of potential ectoine/hydroxyectoine substrate binding proteins of Ehu-type transporters found through bioinformatical analysis. (A-E) Models and structural data are based on the structure of ectoine/hydroxyectoine binding protein EhuB of the EhuABC ABC-type transporter from *S. meliloti* (PDB ID: 2Q88) (Hanekop *et al.*, 2007). (A,B) Comparison of the overall fold of the EhuB substrate binding protein from *S. meliloti* bound to ectoine and of the modelled structures from *Algicoccus marinus* HZ20 (purple) and *M. loti* (light blue). (C-E) Close up of the ectoine binding pocket of the authentic (C) and modelled (D and E) EhuB binding proteins. The ectoine molecule is shown in yellow and residues interacting (or are predicted to interact) with the ligand are shown in orange. (F) Protein sequence alignment of EhuB-type substrate binding proteins in comparison to the structurally characterized EhuB protein from *S. meliloti*. Residues found to be involved in ectoine or hydroxyectoine binding within the crystal structures of *S. meliloti* EhuB (PDB ID: EhuB::ectoine 2Q88, EhuB::hydroxyectoine 2Q89) (Hanekop *et al.*, 2007) are marked with a red dot.

Fig. S3. Modelling and protein sequence alignment of OpuCC-type substrate binding proteins. (A) Modelling is based on the structure of compatible solute binding protein OpuCC of the OpuC ABC-type transporter from *Bacillus subtilis* (PDB ID: 3PPR) (Du *et al.*, 2011). Comparison of the overall fold of OpuCC bound to ectoine from *B. subtilis* and the modeled structure from *Virgibacillus halodenitrificans* (rainbow). (B,C) Zoom into the ectoine binding pocket of the authentic (B) and modeled (C) OpuCC binding proteins. The ectoine protein is shown in yellow and residues interacting (or are predicted to interact) with the ligand are represented as sticks. Modelling was performed using the SWISS Model online tool (Waterhouse *et al.*, 2018). (D) Protein sequence alignment of OpuCC-type substrate binding proteins in comparison to the closely related and structurally characterized substrate binding proteins OpuBC and OpuCC proteins from *B. subtilis* (Du *et al.*, 2011, Pittelkow *et al.*, 2011). Residues found to be involved in ectoine binding within the crystal structure of *B. subtilis* OpuCC (PDB ID: 3PPR) are marked with a red dot.

Fig. S4. Modelling and protein sequence alignment of ProX-type substrate binding proteins. (A) Modelling and structural data are based on the structure of compatible solute binding protein ProX of

the ProU ABC-type transporter from *Escherichia coli* (PDB ID: 1R9L) (Schiefner *et al.*, 2004). Comparison of the overall fold of ProX protein from *E. coli* bound to glycine betaine and the modelled structure of the corresponding ProX-type substrate binding protein from *Vibrio anguillarum* (rainbow). (B,C) Zoom into the glycine betaine binding pocket of the authentic (B) and modelled (C) ProX-type binding proteins. The glycine betaine molecule is shown in green and residues interacting (or are predicted to interact) with the ligand are represented as sticks. Modeling was performed using the SWISS Modell online tool (Waterhouse *et al.*, 2018). (D) Protein sequence alignment of ProX-type substrate binding proteins in comparison to the functionally and structurally characterized ProX protein from *E. coli* (Schiefner *et al.*, 2004). Residues involved in glycine betaine binding within the crystal structure of *E. coli* ProX protein (PDB ID: 1R9L) are marked with a red dot.

Fig. S5. Modelling and protein sequence alignment of TeaA-type substrate binding proteins. (A) Modeling and structural data are based on the structure of ectoine/hydroxyectoine binding protein TeaA of the TeaABCD TRAP-type transporter from *Halomonas elongata* (PDB ID: 2VPN) (Kuhlmann *et al.*, 2008). Comparison of the overall fold of the TeaA protein from *H. elongata* bound to ectoine and the modelled structure of a TeaA-type protein from *Alcanivorax borkumensis* (blue). (B,C) Zoom into the ectoine binding pocket of the authentic (B) and modelled (C) TeaA binding proteins. Ectoine is shown in yellow and residues interacting (or are predicted to interact) with the ligand are represented as sticks. Modelling was performed using the SWISS Model online tool (Waterhouse *et al.*, 2018). (D) Protein sequence alignment of TeaA-type substrate binding proteins in comparison to the functionally and structurally characterized TeaA protein from *H. elongata* (Kuhlmann *et al.*, 2008). Residues involved in ectoine or hydroxyectoine binding within the crystal structures of *H. elongata* TeaA protein (PDB ID: TeaA::ectoine 2VPN; TeaA::hydroxyectoine 2VPO) are marked with a red dot.

Fig. S6. Modelling and protein sequence alignment of BCCT-type transporters. (A,B) Modeling and structural data are based on the structure of the BCCT-type transporter BetP from *C. glutamicum* (PDB ID: 2WIT) (Ressl *et al.*, 2009). Comparison of the overall fold of BetP bound to glycine betaine from *C. glutamicum* and the modeled structure from a BCCT-type protein from *Vibrio alginolyticus* (rainbow). (C,D) Zoom into the substrate binding pocket of the modelled (C) and authentic (D) BetP-type transporter. (C) Ectoine (yellow) and hydroxyectoine (pink) were docked into the modeled BCCT-type transporter. (D) Glycine betaine bound in the substrate binding pocket of BetP is shown in green and residues interacting (or are predicted to interact) with the ligand are represented as sticks. Modeling was performed using Alphafold2 and docking using a combination of AutoDock and DrugScore2018 (Goodsell et al., 1996; Sotriffer et al., 2002; Dittrich et al., 2019; Jumper et al., 2021). (D) Protein sequence alignment of BCCT-type transporters in comparison to the functionally and structurally

characterized BetP protein from *C. glutamicum* (Ressl et al., 2009). Residues involved in glycine betaine binding within the crystal structure of *C. glutamicum* BetP (PDB ID: BetP::glycine betaine 2WIT) are marked with a red dot, residues required for the co-transport of Na^+ are labelled with a blue dot.

Fig. S7. Protein sequence alignment of EctI-type importers. Protein sequence alignment of EctI-type transporters that belong to the SSS-family whose genes are found in the gene neighborhood of ectoine/hydroxyectoine biosynthetic genes.

Fig. S8. Protein sequence alignment of EctU-type importers. Protein sequence alignment of EctU-type uptake systems belonging to the MFS family whose genes are found in the gene neighborhood of ectoine/hydroxyectoine biosynthetic genes. The alignment also includes the functionally characterized ProP transporter for compatible solute from *E. coli* (including ectoine and hydroxyectoine) as a reference sequence (MacMillan *et al.*, 1999).

Fig. S9. Modelling of the potential ectoine exporter EctX. (A) The putative ectoine exporter EctX was modeled using Alphafold 2 (Jumper et al, 2021). (B) Structural comparison with already published protein structures using PDBefold revealed high similarity to the multidrug MFS-type efflux system MdfA from *E. coli* (Heng *et al.*, 2015). The overall structure is shown in grey. The chloramphenicol bound in the substrate binding site of the MdfA transporter (8PDB ID: 4ZOW). (C) Overlay of the modelled EctX transporter and of the crystal structure the MdfA transporter from *E. coli*. The RMSD value calculated by PyMol (Delano, 2002) is 5.44 Å. The ligand binding site is highlighted with a red circle.

Fig. S10. Protein sequence alignment of EctX-related MFS-type exporters. Protein sequence alignment of potential EctX-type export systems for compatible solutes found in the gene neighborhood of ectoine/hydroxyectoine biosynthetic gene clusters.

Fig. S11. Protein sequence alignment of MscS/YbdG-type mechanosensitive channels. Protein sequence alignment of the *Novosphingobium* sp. LH128 YbdG-type mechanosensitive channel protein compared to the functionally characterized MscS and YbdG mechanosensitive channels from *E. coli* (Amemiya *et al.*, 2019, Schumann *et al.*, 2010, Wang *et al.*, 2008).

Table S1. Plasmids used in this study.

Table S2. Bacterial strains used in this study.

Table S3. Primers used in this study.

Legends to Figures:

Fig. 1. Overview and classification of ectoine/hydroxyectoine biosynthetic gene clusters associated with different transporter genes. (A) Chemical formulas of ectoine [(4S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] and hydroxyectoine [(4S,5S)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid]. (B) Schematic overview of the genome context of selected ectoine/hydroxyectoine biosynthetic gene clusters and their associated transporter genes.

Fig. 2. Transcriptional analysis of the *ect* gene cluster from *Novosphingobium* sp. LH128 and *H. neptunium*. Genetic organization of the *ect* gene clusters from *Novosphingobium* sp. LH128 (A) and *H. neptunium* (D). The intergenic regions of the two gene clusters were amplified by reverse-transcription PCR (RT-PCR) (B) and (E), and the sizes of the amplified DNA-regions are indicated in (A) and (D) by red bars. (C) and (F) Osmoregulated transcription of the *ectA, ectD, ectX, ectU, ectI, and ybdG* genes. In the case of *Novosphingobium* sp. LH128, the transcription of the *ybdG* gene was also analyzed via RT-qPCR. Total RNA was isolated from cells grown in medium without and with the addition of 0.6 M (for *Novosphingobium* sp. LH128), or 0.5 M (for *H. neptunium*) NaCl respectively. The relative expression of each gene under the tested growth conditions was determined by using the *gyrB* transcript levels in *H. neptunium* and *Novosphingobium* sp. LH128 cells grown under optimal or elevated salt concentrations as the reference standard. Induction values are expressed as the log 2 of the ratio of the value determined for the salt-stressed cells over the value determined for the non-stressed control.

Fig. 3. Salt tolerance of *H. neptunium* and *Novosphingobium* sp. LH128, and synthesis of hydroxyectoine. The growth yield (OD_{578}) of *H. neptunium* (A) and of *Novosphingobium* sp. LH128 (B) cultures was monitored after 24 h of incubation in medium supplemented with increasing NaCl concentrations. (C) and (D) The intracellular hydroxyectoine content of *H. neptunium* (C) and *Novosphingobium* sp. LH128 (D) cells grown in medium with increasing NaCl concentrations was determined by HPLC analysis. No ectoine could be detected in these cells.

Fig. 4. Assessment of transporter activities and substrate profiles of EctI, EctU, and EctX. Growth yield (OD_{578}) of cells the *E. coli* strain MKH13 lacking the osmoprotectant uptake systems ProP and ProU (Haardt *et al.*, 1995) and constitutively expressing the heterologous *ectI*, *ectU* and *ectX* genes from a *lac* promoter present on the used low-copy number recombinant plasmid were monitored. The cells were grown for 24 h (EctI) or 48 h (EctU, EctX) in a minimal medium supplemented without, or with, the addition of 0.8 M NaCl. The media contained 1 mM of the indicated compatible solute. The data shown are the mean and standard error mean of at least four biological replicates.

Fig. 5. Transport characteristics of the EctI and EctU transporters. Growth rates of *E. coli* MKH13 (*proP proU*) cells expressing either the EctU importer from *Novosphingobium* sp. LH128 (A,B), or the EctI importer from *H. neptunium* (C,D,E) were plotted against the concentration of the indicated compatible solutes present in the growth medium. The corresponding *ectI* and *ectU* genes were constitutively expressed from the *lac* promoter present in the backbone of the low-copy number plasmids pANDA (*ectU*$^+$), and pARO12 (*ectI*$^+$), respectively. Cells were grown in a minimal medium (MMA) containing 0.8 M NaCl and increasing concentrations (from 10 to 1000 µM) of the indicated compatible solutes. The growth rates at different compatible solute concentrations were fitted according to the Monod equation (Monod, 1949). The data shown is the mean and standard error mean of at least six biological replicates.

Fig. 6 Modeling and docking of the EctI ectoine/hydroxyectoine importer from *H. neptunium*. (A) Predicted overall fold of the EctI protein. (B) Docking of ectoine (yellow) and hydroxyectoine (magenta) in the putative substrate binding site of the EctI transporter. Residues that can potentially interact with the substrates are highlighted in orange. (C) Crystal structure of the structural homolog of the EctI transporter, SiaT in complex with its substrate N-acetylneuraminic acid (PDB ID: 5NVA) (Wahlgren *et al.*, 2018). (D) Overlay of the EctI structural model with the experimentally determined crystal structure of SiaT.

Fig. 7 Modelling and docking of the EctU ectoine/hydroxyectoine importer from *Novosphingobium sp.LH128*. (A) Predicted overall fold of the EctU protein. (B) Docking of ectoine (yellow) and hydroxyectoine (magenta) in the putative substrate binding site of the EctU transporter. Residues that can potentially interact with the substrates are highlighted in orange. (C) Crystal structure of the structural homolog of the EctU transporter, XylE in complex with its substrate D-xylose (PDB ID: 4GBY) (Sun *et al.*, 2012). (D) Overlay of the EctU structural model with the experimentally determined crystal structure of XylE. The position of the predicted substrate binding site is circled in (C) and (D).

Fig. 8. Analysis of the putative ectoine/hydroxyectoine exporter EctX from *Novosphingobium* sp. LH128. (A) Growth yield (OD_{578}) of the ProU$^+$ ProP$^+$ *E. coli* wild type strain MC4100 either harboring the empty expression vector pTrc99a or plasmid pLC108 (*ectX*$^+$) where the expression of this gene is controlled by a LacI/IPTG-responsive *lac* promoter. The cells were grown for 48 h in MMA either without additional salt or with 0.8 M NaCl. When indicated, high salinity growth media were supplemented with 1 mM of various compatible solute. All cultures were grown in the presence of 0.3 mM IPTG. The data shown represent the mean and standard error mean of six biological replicates. (B) and (C) Growth curves (OD_{578}) of *E. coli* wild type strain MC4100 harboring either the empty expression

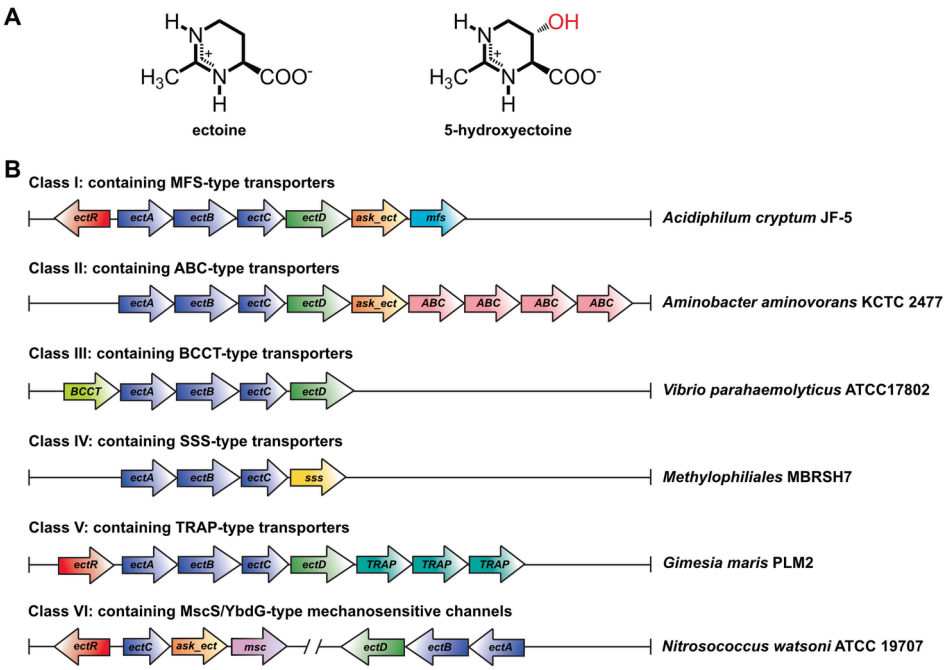
vector pTrc99a (B) or plasmid pLC108 (*ectX*$^{+}$) (C). The cells were grown for 72 h in 0.8 M NaCl MMA medium supplemented with 1 mM of the indicated compatible solute and 0.3 mM IPTG. (D) and (E) Growth curves (OD_{578}) of the *E. coli* wild type strain MC4100 harboring either the empty expression vector pTrc99a (blue) or plasmid pLC108 (*ectX*$^{+}$) (red). The cells were cultivated in MMA containing 0.8 M NaCl and were supplemented with 1 mM hydroxyectoine either in the absence (D) or presence (E) of 0.3 mM IPTG.

Fig. 9. Modelling and functional assessment of the MscS/YbdG-type mechanosensitive channel from *Novosphingobium* sp. LH128. (A) Scheme of the overall fold of a monomer of the *E. coli* MscS mechanosensitive channel (PDB ID: 6PWP) protein (Reddy *et al.*, 2019). (B) Scheme of the overall fold of a monomer of the modelled YbdG-type mechanosensitive channel protein from *E. coli* and (C) from *Novosphingobium* sp. LH128. These models were generated using AlphaFold2 (Jumper *et al.*, 2021). The *E. coli* MscS and YbdG proteins form heptameric complexes (Edwards *et al.*, 2012, Pliotas *et al.*, 2015). (D) Survival (%) of cells of the *E. coli* wild-type strain FRAG1 possessing all currently known mechanosensitive channels and its mutant derivatives MJF641 that lacks all seven of them (Edwards *et al.*, 2012). Cells of strains FRAG1 and its channel-less mutant derivative MJF641 carried either the expression vector pTrc99a, or the *ybdG*-expression plasmid pLC19 were grown in a minimal medium in the presence of 0.3 M NaCl and 1 mm IPTG until they reached an OD_{578} of about 0.35. At this point, the cells were subjected to an osmotic down-shock from 0.3 M NaCl to 0 M NaCl as described (Levina *et al.*, 1999). The number of cells surviving this challenge were determined by spotting aliquots of appropriate dilutions onto LB-agar plates. The data shown represent the mean and standard error mean of three independent biological replicates.

Table 1. 523 genes encoding potential transporters and channels are found in 415 ectoine biosynthetic gene clusters.

Class	Transporter family	Homolog	Abundance	Sequence identity [%]
I	MFS	ProP	117	39-50
		efflux systems*	56	24-37
II	ABC	EhuB	134	33-74
		ProX	83	26-67
		OpuBC/OpuCC	3	57
III	BCCT	BetP/EctT	67	33-42
IV	SSS	PutP/OpuE	32	20-30
V	TRAP	TeaA	8	27-33
VI	Msc	MscS, YbdG	23	18-54

*YcaD, MdfA, MdtG



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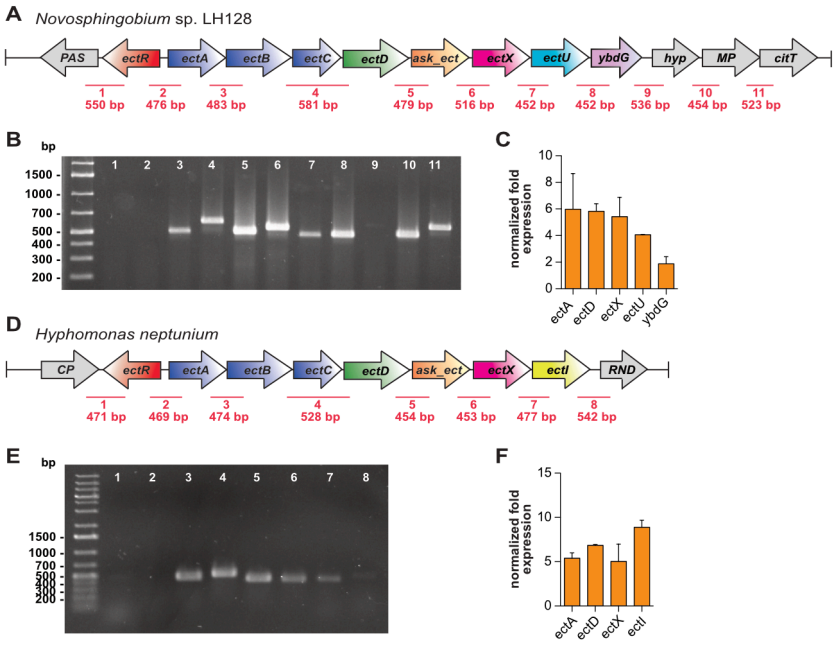

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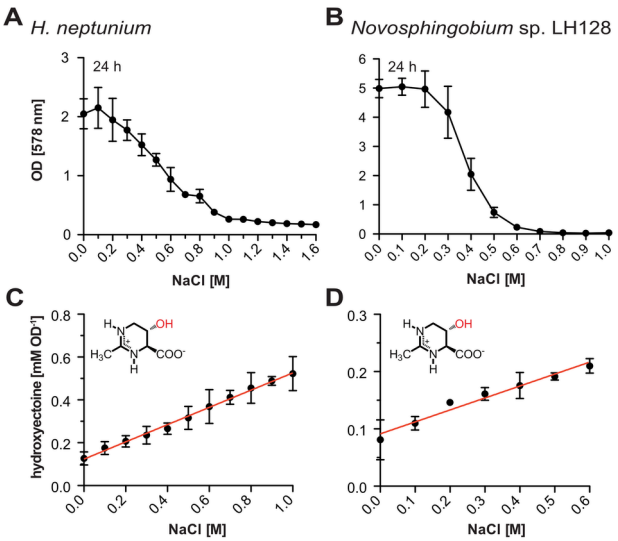

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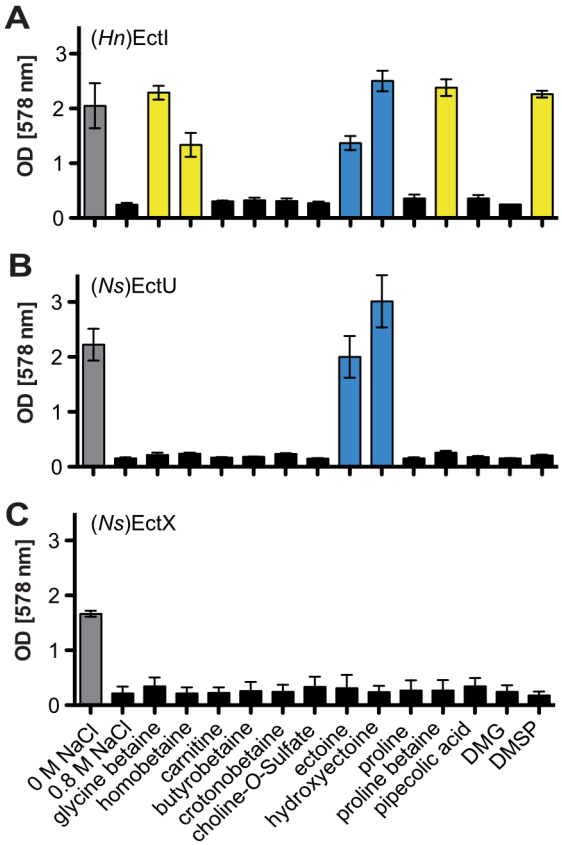

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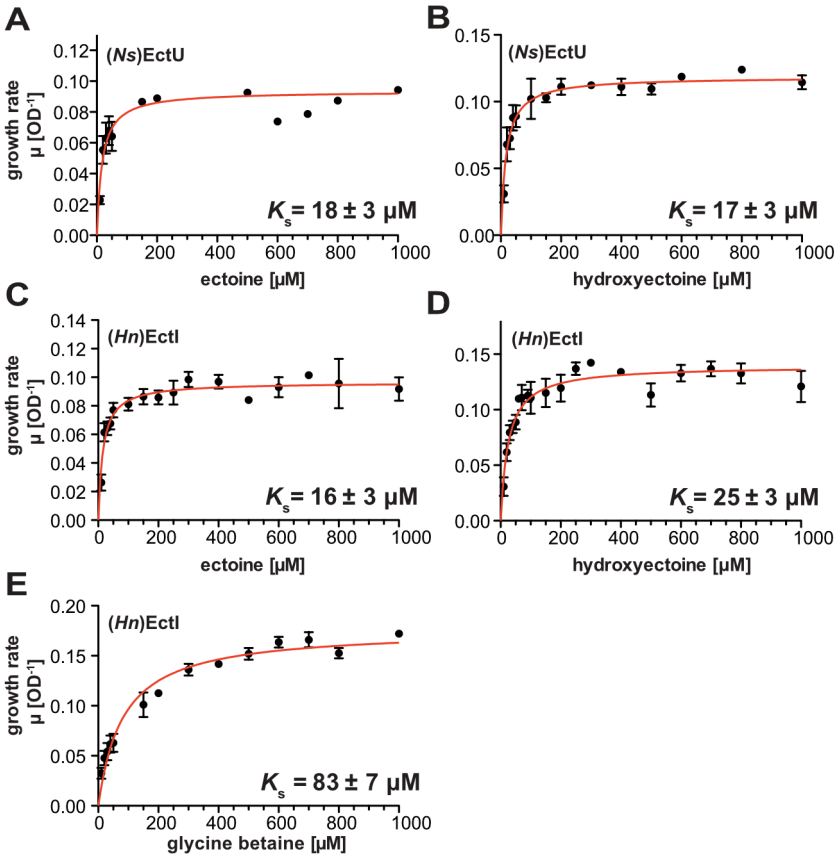

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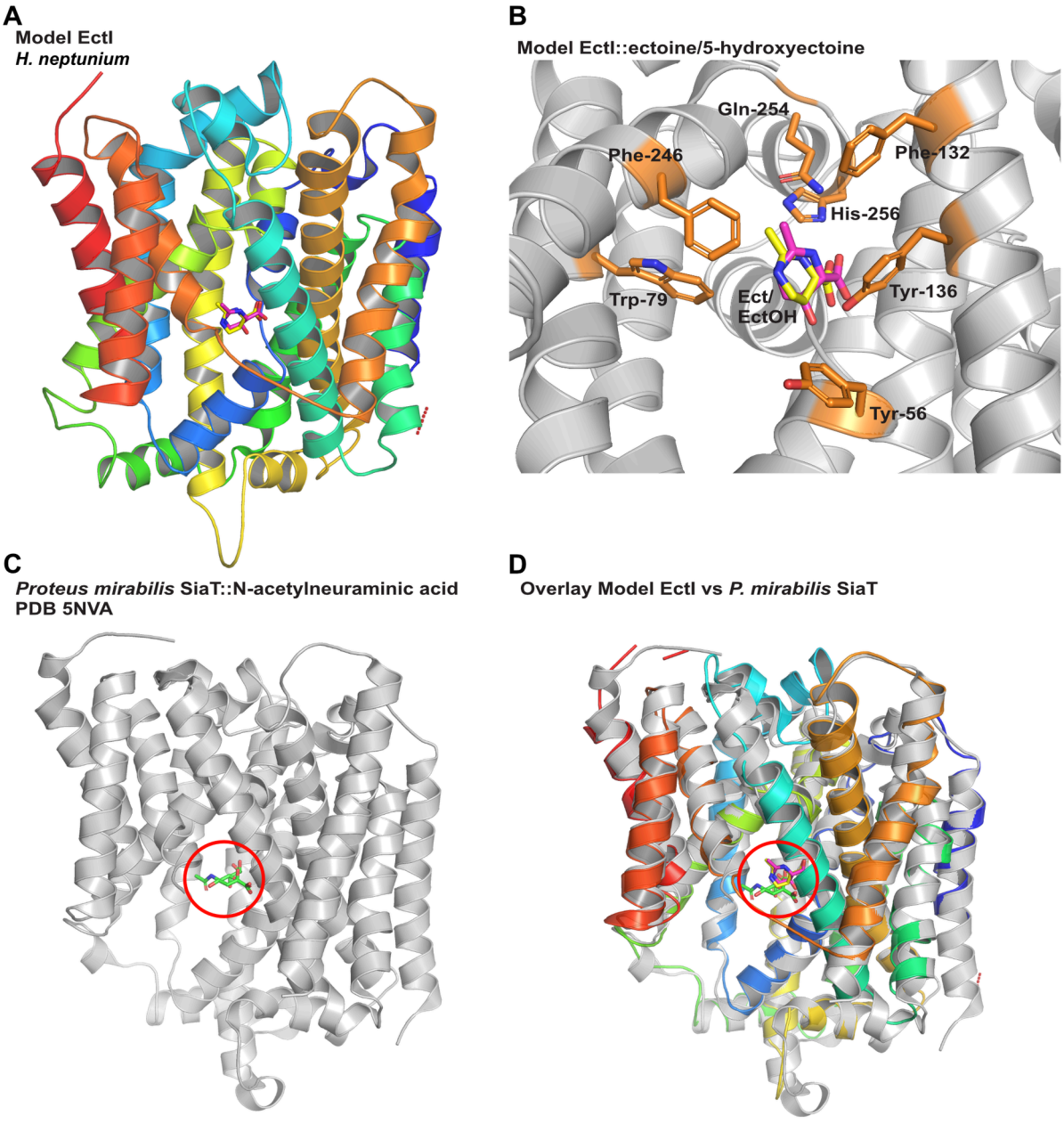

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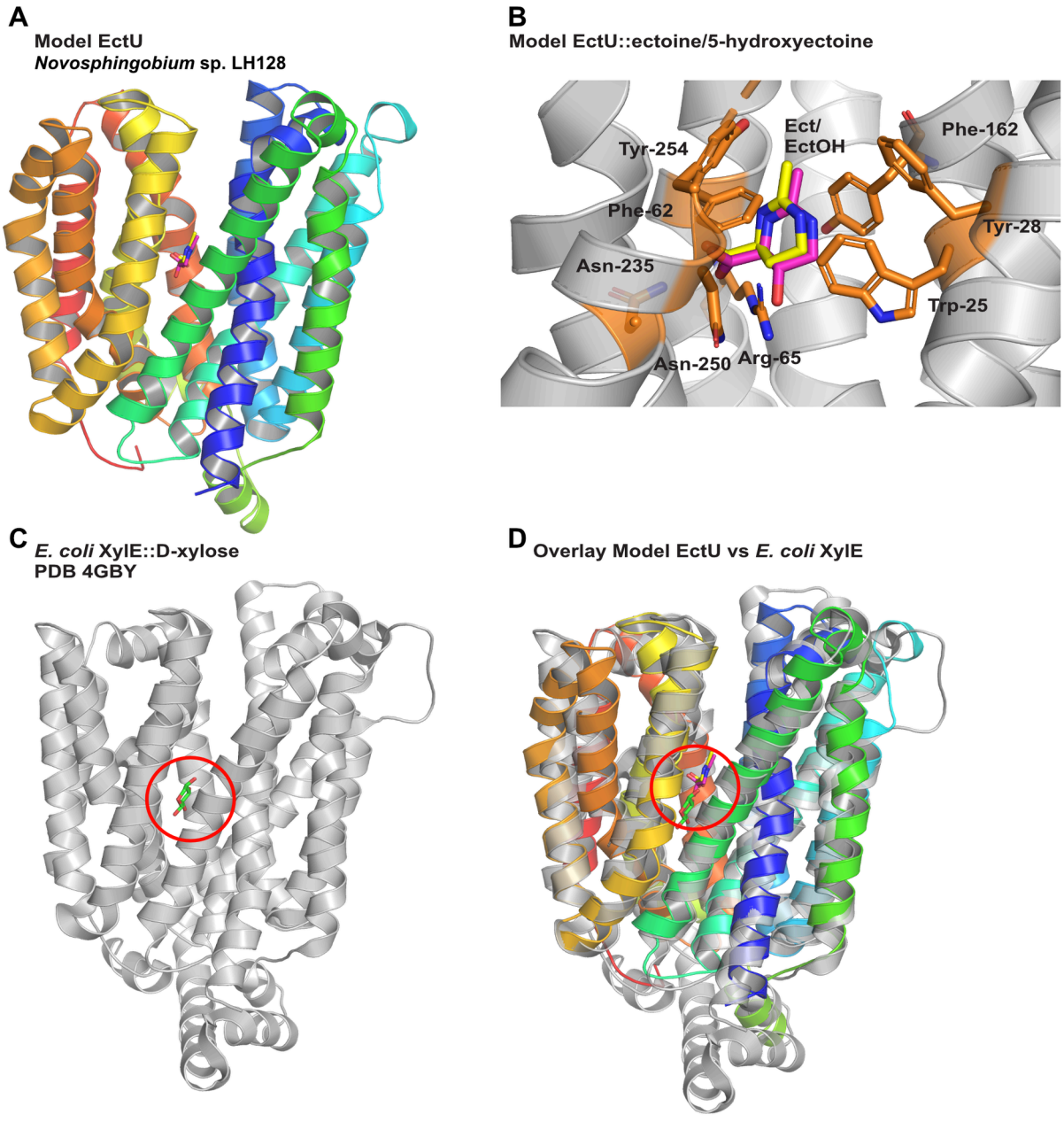

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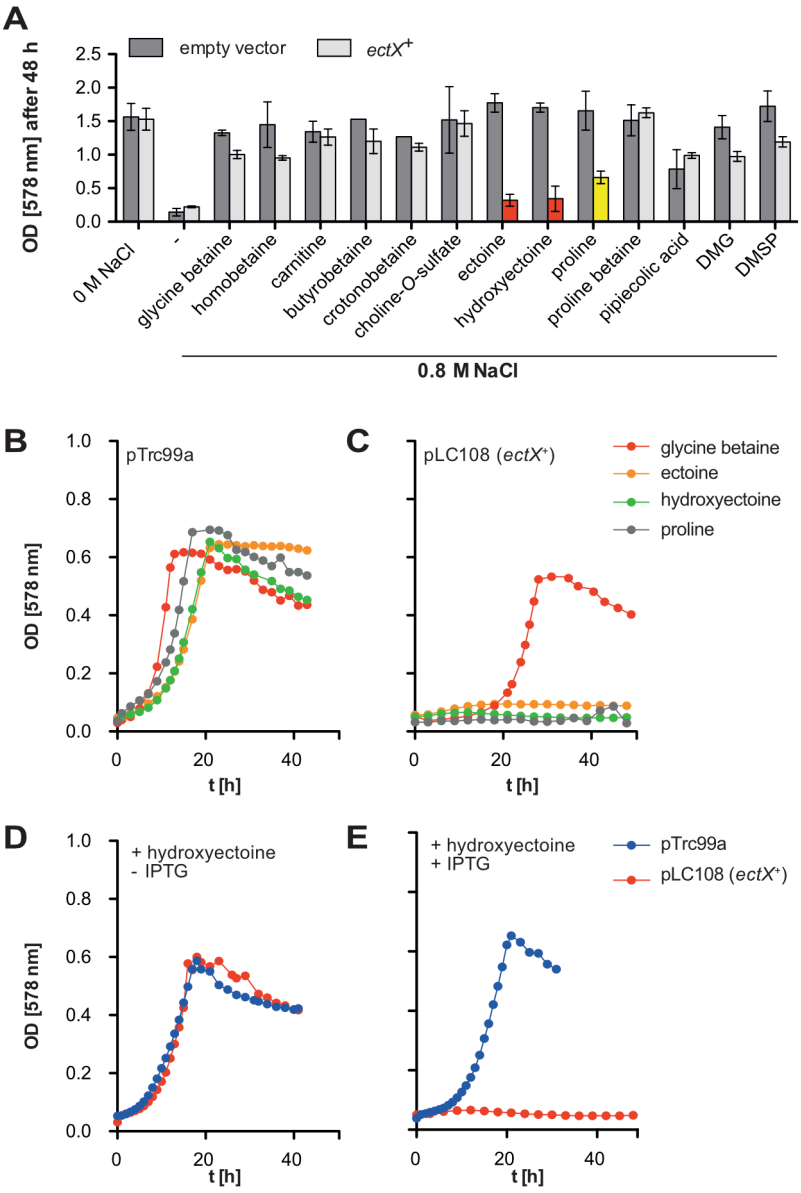

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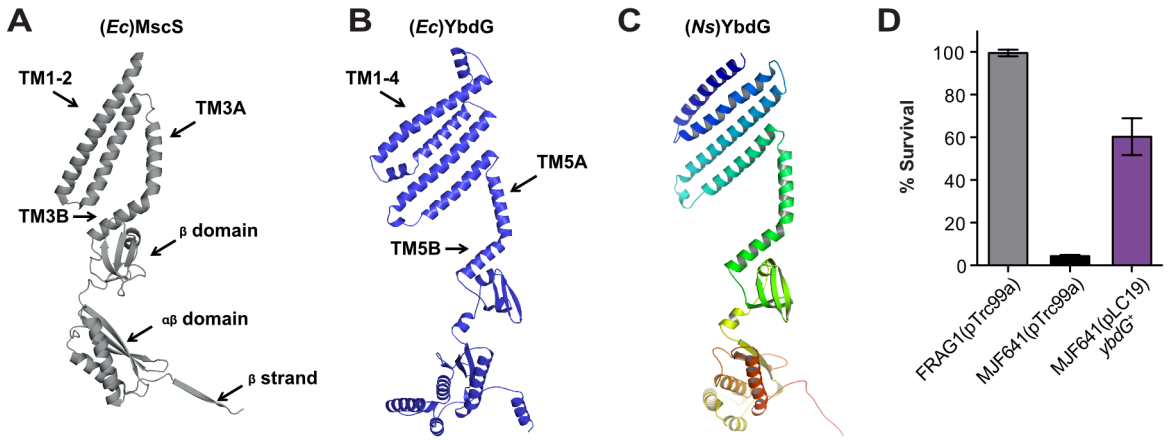

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