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MicroReview

The BCCT family of carriers: from physiology to crystal structure

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Summary

Increases in the environmental osmolarity are key determinants for the growth of microorganisms. To ensure a physiologically acceptable level of cellular hydration and turgor at high osmolarity, many bacteria accumulate compatible solutes. Osmotically controlled uptake systems allow the scavenging of these compounds from scarce environmental sources as effective osmoprotectants. A number of these systems belong to the BCCT family (betaine-choline-carnitine-transporter), sodium- or proton-coupled transporters (e.g. BetP and BetT respectively) that are ubiquitous in microorganisms. The BCCT family also contains CaiT, an L-carnitine/γ-butyrobetaine antiporter that is not involved in osmotic stress responses. The glycine betaine transporter BetP from *Corynebacterium glutamicum* is a representative for osmoregulated symporters of the BCCT family and functions both as an osmosensor and osmoregulator. The crystal structure of BetP in an occluded conformation in complex with its substrate glycine betaine and two crystal structures of CaiT in an inward-facing open conformation in complex with L-carnitine and γ-butyrobetaine were reported recently. These structures and the wealth of biochemical data on the activity control of BetP in response to osmotic stress enable a correlation between the sensing of osmotic stress by a transporter protein with the ensuing regulation of transport activity. Molecular determinants governing the high-affinity binding of the compatible solutes by BetP and CaiT, the coupling in symporters and antiporters, and the osmoregulatory properties are discussed in detail for BetP and various BCCT carriers.

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Introduction

One of the key parameters affecting the survival and growth of microorganisms are changes in the osmotic conditions of their habitat (Kempf and Bremer, 1998; Wood *et al.*, 2001; Poolman *et al.*, 2002; Altendorf *et al.*, 2009; Hagemann, 2010). Exposure of the cell to either hypo- or hyper-osmotic surroundings triggers rapid water fluxes across the semi-permeable cytoplasmic membrane (Fig. 1), whose direction is determined by the differential in osmotic potential between the cell's interior and the environment (Wood, 1999; Bremer and Krämer, 2000; Altendorf *et al.*, 2009). Both the osmotically instigated water fluxes and the resulting changes in turgor, an intracellular hydrostatic pressure, impinge on many physiological processes (Record *et al.*, 1998; Wood, 1999). AqpZ-type aquaporins that are present in a substantial number of microorganisms mediate accelerated water fluxes across the cytoplasmic membrane in response to osmotic gradients (Tanghe *et al.*, 2006). However, these types of water channels are not essential for water fluxes across the cell membrane in bacteria. Most importantly, no single microorganism can actively transport water into or out of the cell. But microorganisms can actively influence the direction of water fluxes across the cell membrane in response to hyper- or hypo-osmotic conditions. They do so by indirectly determining the scale of the osmotically driven water fluxes across the cytoplasmic membrane by actively adjusting the osmotic potential of their interior via active control of their ion and organic solute pools (Fig. 1).

Environmental osmotic up- and downshifts and compensatory microbial reactions

Exposure of a microbial cell to a hypo-osmotic surrounding triggers rapid water influx (Fig. 1). In extreme cases,

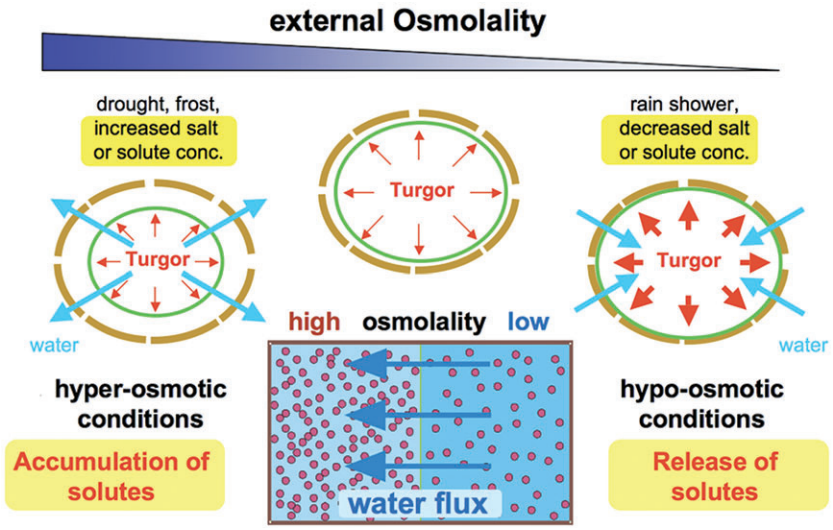

Fig. 1. Osmotically driven water fluxes across the cytoplasmic membrane of a microbial cell exposed either to hyper- or hypo-osmotic surroundings.

water entry can drive up turgor to such magnitudes that the stress-bearing peptidoglycan sacculus can no longer cope with it and the cell is threaten to bursts (Booth *et al*., 2007). To avoid such a catastrophic event, mechanosensitive channels are transiently opened as safety valves through which both solutes and solvents can pass rapidly (Perozo, 2006; Martinac *et al*., 2008; Anishkin and Sukharev, 2009). Hence, the cell can reduce the osmotic potential of its interior within seconds and thereby curbs water influx and reduces turgor; as a consequence, cell lysis is prevented (Booth *et al*., 2007).

Exposure of a microbial cell to hyper-osmotic conditions elicits water efflux (Fig. 1), thereby causing dehydration of the cytoplasm, a reduction or even collapse of turgor, and eventually growth arrest (Record *et al*., 1998). The slowing of cell growth and finally growth arrest are strongly correlated with the diminished amounts of free cytoplasmic water (Cayley and Record, 2003). The cell can increase the content of free cytoplasmic water by raising the osmotic potential of its interior via accumulation of either ions or organic osmolytes (Kempf and Bremer, 1998; Wood, 1999; Bremer and Krämer, 2000; Wood *et al*., 2001; Oren, 2008; Altendorf *et al*., 2009; Hagemann, 2010).

Properties of compatible solutes

Many microorganisms accumulate a selected class of organic osmolytes, the compatible solutes, to fend off the detrimental effects of high osmolarity on water content and cell physiology (Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood *et al*., 2001; Empadinhas and da Costa, 2008). Compatible solutes are operationally defined as organic osmolytes that can be accumulated to exceedingly high intracellular levels without impairing vital functions of the cell (Kempf and Bremer, 1998; Burg and Ferraris, 2008; Empadinhas and da Costa, 2008). These solutes have biophysical and biochemical properties that distinguish them from other types of organic compounds, and they have been specifically selected during the course of evolution as cytoprotectants in all three kingdoms of life (Yancey *et al*., 1982; 2005). A hallmark of compatible solutes is their preferential exclusion from the immediate hydration shell of proteins (Arakawa and Timasheff, 1985) due to unfavourable interactions between the osmolytes and the protein backbone (Bolen and Rose, 2008). This uneven distribution of compatible solutes in the cell water generates a thermodynamic driving force that promotes the proper conformation of proteins and the formation of protein assemblies, thereby maintaining their biological functions (Bolen and Baskakov, 2001; Ignatova and Gierasch, 2006; Street *et al*., 2006; Rösgen, 2007; 2009; Capp *et al*., 2009; Street *et al*., 2010). It should be noted, however, that compatible solutes that are ubiquitously used by microorganisms (e.g. proline and glycine betaine) interact differently with protein surfaces (Auton *et al*., 2008).

Microbial transport systems for compatible solutes

Most microorganisms that adopt the 'salt-out' strategy both synthesize and acquire various types of compatible solutes. Osmotically down-shocked or decaying microbial cells (e.g. after viral attack), excretion products of animals and plants (urine and root exudates respectively) and of algae are sources of these compounds (Welsh, 2000). Compatible solutes are found in very low concentrations (typically in the μM or nM range) in the environment. To take advantage of these scarce resources as osmostress protectants, microorganisms use osmotically controlled high-affinity uptake systems (Kempf and Bremer, 1998; Wood, 1999; 2006; Bremer and Krämer, 2000; Wood *et al*.,

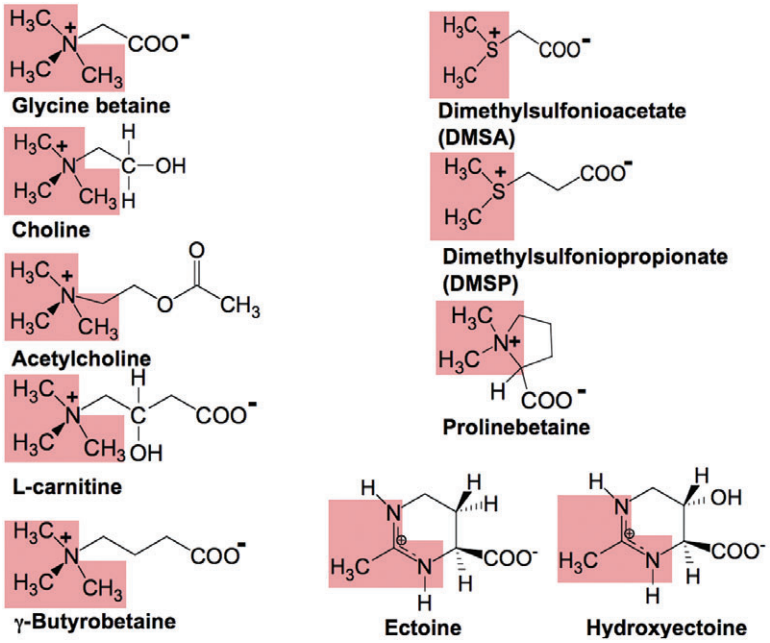

Fig. 2. Known substrates for BCCT carriers. The positively charged nitrogen or sulphur head-groups and the delocalized positive charge present in ectoine and hydroxyectoine are highlighted.

2001; Poolman *et al*., 2004; Krämer, 2009; Hagemann, 2010). The intracellular level to which compatible solutes are amassed is fine-tuned to the degree of osmotic stress perceived by the microbial cell. Microorganisms can accumulate compatible solutes via transport systems against steep concentration gradients, and intracellular concentrations above one molar can be attained (Peter *et al*., 1996; Krämer, 2009). Uptake of compatible solutes by microorganisms is energetically favourable over *de novo* synthesis (Oren, 1999). Furthermore, the uptake of compatible solutes often represses the expression of genes encoding enzymes required for compatible solute synthesis. Transport systems for compatible solutes not only serve to capture these compounds from scarce environmental sources but also serve for the re-import of these compounds leaked from microbial producer cells in high osmolarity surroundings (Lamark *et al*., 1992; Mikkat and Hagemann, 2000; Grammann *et al*., 2002).

Frequently, the transcription of the structural genes encoding compatible solute transporters is upregulated in response to increases in the osmolarity of the environment. Typically, gene expression is rapidly induced after the cells experience an osmotic up-shift, promoter activity being adjusted to the degree of the prevalent osmotic stress (Spiegelhalter and Bremer, 1998; Cheung *et al*., 2003; Nagarajavel *et al*., 2007; Romeo *et al*., 2007; Rosenthal *et al*., 2008; Altendorf *et al*., 2009; Krämer, 2010). Furthermore, the activity of the compatible solute transporter itself is often subjected to osmotic control on the level of activity (Wood, 1999; 2006; van der Heide and Poolman, 2000; van der Heide *et al*., 2001; Poolman *et al*., 2004; Krämer, 2009; Mahmood *et al*., 2009; Romantsov *et al*., 2009). Thus, microbial uptake systems for compatible solutes are not only effective transporters but they can also sense and respond to osmotic stress themselves. This permits a practically instantaneous physiological response by allowing the osmotically stressed cell to accumulate types of solutes that restrict water loss and that are at the same time congruous with its other physiological needs. By sensitive adjustment of both the amount of the transport protein(s) via gene expression and *de novo* protein synthesis and the activity of the transporter itself, the bacterial cell can exert maximum control over its cellular compatible solutes pool.

The preferential exclusion of compatible solutes from protein surfaces is a hallmark of the protective function of these types of solutes (Arakawa and Timasheff, 1985; Bolen and Baskakov, 2001; Ignatova and Gierasch, 2006; Street *et al*., 2006; 2010; Rösgen, 2007; Bolen and Rose, 2008). However, in transport systems for compatible solutes, high-affinity interactions between the substrates and proteins have to take place in order to archive effective import. Crystallographic studies with soluble ligand-binding proteins from six ABC and two TRAP transport systems and the secondary transporters BetP and CaiT have recently provided insight into how such a task can be accomplished (Schiefner *et al*., 2004a,b; Horn *et al*., 2006; Oswald *et al*., 2008; Smits *et al*., 2008; Ressl *et al*., 2009; Schulze *et al*., 2010; Tang *et al*., 2010; Wolters *et al*., 2010). In all these systems, aromatic ligand binding boxes of various architectures recognize the bulky head-groups of glycine betaine, choline, L-carnitine, γ-butyrobetaine and dimethylsulphonioacetate (DMSA) (Fig. 2) via cation-pi interactions. Hydrogen bonds and salt-bridges then fix and orient the various 'tails' of the

different substrates (Fig. 2) within the ligand binding site. Cation-pi interactions also play important roles for the high-affinity binding of ectoine and hydroxyectoine by soluble ligand binding proteins from ABC or TRAP transporters, but due to the chemical features of these compatible solutes (Fig. 2), the architecture of the ligand binding sites is different form those that are used to bind compatible solutes such as glycine betaine, proline betaine and DMSA (Hanekop *et al*., 2007; Kuhlmann *et al*., 2008; Lecher *et al*., 2009).

The types of functionally characterized microbial transporters for compatible solutes include members of the multi-component ABC (e.g. ProU, OpuA and Ehu) and TRAP transporter families (Tea and Ueh) and single-component transport systems such as ProP (H^+/solute symporter; MSF family), OpuE (Na^+/solute symporter; SSSF family) and members of the BCCT family (e.g. BetT, BetP, OpuD). The prototypes of these osmotically controlled uptakes systems have been discovered through diligent genetic and physiological studies with just a few bacterial species (Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood *et al*., 2001; Bremer, 2002; Altendorf *et al*., 2009; Krämer, 2009). However, even a cursory inspection of the more than 1000 microbial genome sequences available today shows that compatible solute uptake systems related to those mentioned above are found widely in the domain of the *Bacteria* and of the *Archaea*.

Three transporters have been studied in detail by biochemical, biophysical and genetic approaches with the aim of unravelling the mechanism(s) and interplay of their osmoregulatory and transporter function. These are the ABC transporter OpuA from *Lactococcus lactis*, the H^+/solute symporter ProP from *Escherichia coli* and the BCCT carrier BetP from *Corynebacterium glutamicum* (van der Heide and Poolman, 2000; van der Heide *et al*., 2001; Poolman *et al*., 2004; Culham *et al*., 2008a,b; Krämer, 2009; Mahmood *et al*., 2009).

The BCCT carrier BetP from *C. glutamicum* occupies a special position within this group of osmoregulatory compatible solute transport systems because a well-resolved crystal structure of the BetP protein with bound glycine betaine and sodium ligands has been reported (Ressl *et al*., 2009). Most recently, three X-ray structures of another BCCT carrier, the L-carnitine/γ-butyrobetaine antiporter CaiT from *E. coli* and *Proteus mirabilis* was solved in complex with its substrate L-carnitine and γ-butyrobetaine (Schulze *et al*., 2010; Tang *et al*., 2010). The CaiT proteins from these two organisms are not involved in osmostress responses and instead mediate L-carnitine : γ-butyrobetaine antiport under unaerobic growth conditions (Jung *et al*., 2002).

The ligand-bound crystal structures of the BCCT carriers BetP (Ressl *et al*., 2009) and CaiT (Schulze *et al*., 2010; Tang *et al*., 2010) reveal common characteristics with respect to their overall fold, their topological organization with the cytoplasmic membrane and the transport function of these proteins. However, distinct features became apparent from these crystal structures that build the foundation for the osmotic control of the transport activity of BetP. Using the structural data of both the BetP and CaiT transporters and a wealth of biochemical data available for BetP (Ott *et al*., 2008; Krämer, 2009; Ressl *et al*., 2009) and CaiT (Jung *et al*., 2002; Schulze *et al*., 2010) as a point of reference, we will evaluate features for osmotic activity control of BetP to assess the physiological roles of various microbial BCCT carriers.

The BCCT carriers: discovery and general features

The first member (BetT) of the subsequently christened BCCT transporter family was described almost 20 years ago by Arne R. Strom and co-workers in connection with the genetic analysis of the osmoregulatory choline-to-glycine betaine synthesis pathway of *E. coli* (Lamark *et al*., 1991). BetT was shown to mediate the high-affinity uptake of choline, the biosynthetic precursor for the compatible solute glycine betaine. An L-carnitine transporter protein (CaiT) related to the choline transporter BetT was subsequently discovered when the genes involved in L-carnitine metabolism in *E. coli* were studied (Eichler *et al*., 1994). The simultaneous discoveries of the OpuD transporter from *Bacillus subtilis* (Kappes *et al*., 1996) and BetP from *C. glutamicum* (Peter *et al*., 1996), both involved in osmotically controlled glycine betaine uptake, led then Kappes *et al*., (1996) to propose that the aforementioned choline, L-carnitine and glycine betaine transporters form a new transporter subfamily with substrate specificity for various types of trimethylammonium compounds (Fig. 2).

Using the substrates of the BetT, CaiT, OpuD and BetP transporters as a point of reference, Milton Saier's laboratory subsequently grouped these transporters into the BCCT family (betaine-choline-carnitine-transporter) (Saier, 2000) and placed them into sub-group 2.A.15 of the International Union of Biochemistry and Molecular Biology-approved transporter classification (TC) system (http://www.tcdb.org) (Saier *et al*., 2009). BCCT carriers comprise uptake systems that are energized either by proton-motive-force-driven (e.g. BetT) or sodium-motive-force-driven (e.g. BetP) symport, or more rarely, by substrate : product antiport (e.g. CaiT) (Table 1).

It was originally thought that a unifying feature of BCCT carriers is the transport of substrates with quaternary ammonium groups [$R\text{-}N^+(CH)_3)_3$] such as choline, L-carnitine and glycine betaine (Kappes *et al*., 1996; Saier, 2000) (Fig. 2). In recent years, several new substrates for BCCT carriers have been discovered. Of these,

Table 1. Functionally characterized members of the BCCT family.

Transporter [citation]	*Organism* Accession number	Substrate	Predicted driving force/direction	Total number (aa)	Sequence identity to BetP (%)	N-terminal domain (aa)	C-terminal domain (aa)
BetP [1]	*Corynebacterium glutamicum* CAA63771.1	GB	smf/symport	595	100	58	50
OpuD [2]	*Bacillus subtilis* AAC44368.1	GB	smf/symport	512	43	4	24
BetL [3]	*Listeria monocytogenes* AAD30266.1	GB	smf/symport	507	41	3	20
BetH [4]	*Halobacillus trueperi* AAT3760.1	GB	smf/symport	505	41	3	18
BetM [5]	*Marinococcus halophilus* AAQ67247.1	GB	smf/symport	493	38	3	7
ButA [6]	*Tetragenococcus halophila* AAP06751.1	GB	smf/symport	608	34	51	70
OpuD [7, 21]	*Vibrio cholerae* AAF944381.1	GB	smf/symport	539	33	34	15
BetT [8]	*Haemophilus influenza* AAC23352.1	?[a]	?	669	32	7	174
BetT [9]	*Aphanothece halophytica* BAF03065.1	GB	smf/symport	564	32	36	38
BetP [7]	*Vibrio parahaemolyticus* BAC60168.	GB	smf/symport	523	32	41	14
BetU [10]	*Escherichia coli* AAQ10261.1	GB	smf/symport	667	31	17	162
BetS [11]	*Sinorhizobium meliloti* AAL37253.1	GB/PB	smf/symport	706	31	52	167
DddT [12]	*Psychrobacter* sp. ACY02893.1	GB/DMSP	smf/symport	550	28	25	17
LcoP [13]	*Corynebacterium glutamicum* CAF20998.1	E/GB	smf/symport	630	37	43	96
EctT [14]	*Virgibaccilus pantothenticus* AAL16076.1	E/HE	smf/symport	501	36	6	11
EctM [5]	*Marinococcus halophilus* AAQ67247.1	E/HE	smf/symport	493	33	10	25
EctP [15]	*Corynebacterium glutamicum* CAA04760.1	E/P/GB	smf/symport	615	29	21	102
BetT [16]	*Pseudomonas syringae* ZP_03399554	Ch/ACh	pmf/symport	664	30	14	160
BetT [17]	*Escherichia coli* CAQ30790.1	Ch	pmf/symport	677	30	14	175
CudT [18]	*Staphylococcus xylosus* AAD23898	Ch	pmf/symport	540	30	7	36
CaiT [19]	*Escherichia coli* CAQ30560.1	CT/γ-BB	Substrate: product antiport	504	25	10	5
PmCaiT [20]	*Proteus mirabilis* YP_002152355.1	CT/γ-BB	Substrate: product antiport	514	25	15	10

a. The BetT protein from *Haemophilus influenzae* was suggested to function in the uptake of choline [Fan *et al.* (2003) *Mol Microbiol* **50:** 537–48], but the actual substrate has not been unambiguously determined.

References for Table 1:

[1] Peter *et al.* (1996) *J Bacteriol.* **178:** 5229–5234; [2] Kappes *et al.* (1996) *J Bacteriol.* **178:** 5071–5079; [3] Sleator *et al.* (1999) *Appl Environ Microbiol* **65:** 2078–2083; [4] Lu *et al.* (2004) *FEMS Microbiol Lett* **235:** 393–399; [5] Vermeulen *et al.* (2004) *Extremophiles* **8:** 175–184; [6] Baliarda *et al.* (2003) *Curr Microbiol* **47:** 347–351; [7] Naughton *et al.* (2009) *Appl Environ Microbiol* **75:** 2802–2810; [8] Fan *et al.* (2003) *Mol Microbiol* **50:** 537–548; [9] Laloknam *et al.* (2006) *Appl Environ Microbiol* **72:** 6018–6026; [10] Ly *et al.* (2004) *J Bacteriol* **186:** 296–306; [11] Boscari *et al.* (2002) *J Bacteriol* **184:** 2654–2663; [12] Todd *et al.* (2010) *Environ Microbiol* **21:** 327–343: [13] Steger *et al.* (2004) FEBS Lett **573:** 155–160; [14] A.U. Kuhlmann and E. Bremer, unpubl. data; [15] Weinand *et al.* (2007) *Appl Microbiol Biotechnol* **76:** 701–708; [16] Chen and Beattie (2008) *J Bacteriol* **190:** 2717–2725; [17] Lamark *et al.* (1991) *Mol Microbiol* **5:** 1049–1064; [18] Rosenstein *et al.* (1999) *J Bacteriol* **181:** 2273–2278; [19] Eichler *et al.* (1994) *Mol Microbiol* **13:** 775–786; [20] Schulze *et al.* (2010) *Nature* (in press); [21] Kapfhammer *et al.* (2005) *Appl Environ Microbiol* **71:** 3840–3847.

proline betaine, acetylcholine, dimethylsulphoniopropionate (DMSP) and DMSA all posses fully methylated nitrogen or sulphonium headgroups (Fig. 2) and, thus, conform in a wider sense to the originally suggested chemical features of substrates for BCCT carriers. However, the chemical structures of the compatible solutes ectoine and hydroxyectoine (Fig. 2), substrates for BCCT carriers detected in various microbial species (Table 1), do not match this general scheme. In addition, some BCCT-type transporters also mediate proline

uptake, but usually with rather low affinity. So far, no functionally characterized BCCT carrier has been described to catalyse the uptake of the full range of currently known substrates (Table 1; Fig. 2). However, for some BCCT carriers competition experiments have been carried out that suggest a rather restricted substrate specificity for a given BCCT carrier. For instance, in the halotolerant alakaliphilic cyanobacterium *Aphanothece halophytica*, the BetT-mediated glycine betaine transport (Table 1) was not inhibited by glycine betaine aldehyde, dimethylglycine, sarchosine, choline or proline (Laloknam *et al*., 2006).

Physiological functions of BCCT carriers and transcriptional regulation of their structural genes

The explosion in the number of available genome sequences of *Bacteria* and *Archaea* revealed a widespread distribution of BCCT carriers in the microbial world. Under the entry 'IPR000060 BCCT transporter', the InterPro database (http://www.ebi.ac.uk/interpro/) lists currently about 2200 protein sequences. Most of these protein sequences originate from members of the *Bacteria* and only 22 are from members of the *Archaea*. The InterPro database also records a limited number of BCCT carriers from *Eukarya*, but nothing is known about their function.

Most BCCT carriers have a common hydropathy profile with 12 predicted *trans*-membrane (TM)-spanning segments and exhibit N- and C-terminal extensions of various lengths (Table 1) that are predicted to protrude into the cytoplasm. An important functional role of these N- and C-terminal domains for the control of transport activity and osmotic regulation was first discovered for the glycine betaine transporter BetP (Peter *et al*., 1998a). Subsequent studies with the choline transporters BetT of *E. coli* (Tondervik and Strom, 2007) and *Pseudomonas syringae* (Chen and Beattie, 2008) revealed the contribution of the C-terminal domain for the proper functioning of these BCCT carriers as well. The most conserved part of BCCT carriers comprises TM8 and the connecting loop to TM9 and these segments contain the tryptophane motif that is the signature sequence of BCCT carriers (Kappes *et al*., 1996; Saier, 2000).

In the Pfam database (http://pfam.sanger.ac.uk/) some physiologically uncharacterized BCCT carrier-related proteins are listed, which possess additional domains (Usp, CoaE, TrkA_C) or membrane-spanning segments fused to the canonical 12 TM core structures of BCCT carriers. Twenty-two microbial BCCT carriers have so far been functionally characterized at least to some extent (Table 1). Using the *C. glutamicum* BetP glycine betaine transporter protein as the search template, these 22 proteins exhibit a range from 48% down to 25% identical amino acid residues (Table 1). However, it should be noted that functionally uncharacterized BCCT carriers can readily be found in database searches that are much more closely related to the BetP protein from *C. glutamicum* then those listed in Table 1.

Only two of the functionally characterized transporters of the BCCT family are physiologically not connected with microbial osmotic adjustment processes: the CaiT proteins from *E. coli* and *Proteus mirabilis* (Table 1). The CaiT proteins are unusual members of the BCCT family, since they do not require a sodium- or proton-gradient to fuel transport and instead function according to the substrate : product antiport principle. L-carnitine (Fig. 2) is taken up in *E. coli* via CaiT under anaerobic growth conditions when no other electron acceptor or glucose is present and it is then metabolized to γ-butyrobetaine (Fig. 2) that is subsequently excreted via the CaiT antiporter (Jung *et al*., 2002). The genetic regulation of the *caiTABCDE* gene cluster reflects this physiological role. It is controlled by the globally acting regulators CRP and FNR (both positively acting) and the nucleoid-associated protein H-NS (negatively acting) of *E. coli* and by the carnitine-responsive activator protein CaiF (Buchet *et al*., 1998). A BCCT carrier (DddT) was recently shown to mediate uptake of sulphur-containing compatible solute DMSP (Fig. 2) in a relative of *Halomonas venusta* (Todd *et al*., 2010). DMSP is widespread in marine environments and can be used by microorganisms as an effective osmoprotectant or as a versatile sulphur source (Pichereau *et al*., 1998; Dickschat *et al*., 2010; Vila-Costa *et al*., 2010). DddT-mediated uptake of DMSP in the studied *Halomonas strain* is connected with catabolism since expression of the *dddT* gene is induced by DMSP and two of its metabolites (Todd *et al*., 2010).

The majority (19 out of 22) of the currently functionally characterized BCCT carriers (Table 1) are involved in microbial adjustment to high osmolarity surroundings via uptake of compatible solutes or their biosynthetic precursors. In line with these physiological functions, expression of their structural genes is typically induced in cells grown at high osmolarity. With the exception of the proton-coupled choline transporter BetT from *E. coli* and from *Pseudomonas syringae* and CutT from *Staphylococcus xylosus* (Lamark *et al*., 1991; Rosenstein *et al*., 1999; Tondervik and Strom, 2007; Chen and Beattie, 2008), substrate uptake via BCCT carriers is driven by sodium symport (Table 1). As an example, BetP-mediated import of one molecule of glycine betaine is accompanied by the uptake of two sodium ions (Farwick *et al*., 1995; Peter *et al*., 1996). Since glycine betaine concentration above 1 mol l^{-1} can be attained by *C. glutamicum*, massive amounts of Na^+ ions will be pumped into the cell under osmotic stress conditions. It is apparent that the functioning of Na^+-driven BCCT carrires for osmoprotectants

(Table 1) must be physiologically coupled to the functioning of Na^+ extrusion systems in order to keep the intracellular concentration of this cytotoxic cation low (Padan *et al*., 2005).

Functional data, such as K_M and V_{max} values, sodium- or proton-coupling and a description of their activity regulation are available only for a few of the BCCT carriers systems listed in Table 1. We refer the readers for a detailed account of these topics to the original publication (Table 1). A convenient way to assess the substrate specificity of given BCCT carriers, or the genetic section for BCCT carrier-encoding genes from different microorganisms by functional complementation (Kappes *et al*., 1996; Peter *et al*., 1996; Ly *et al*., 2004), is the use of an *E. coli* K-12 mutant strain (MKH13), which lacks the genes for choline uptake via BetT and glycine betaine synthesis (*betBA*) and that is simultaneously defective in the ProU and ProP compatible solute transport systems (Haardt *et al*., 1995).

It is generally not firmly understood at the molecular level how bacteria sense osmotic stress and how this environmental cue ultimately results in altered gene transcription (Bremer and Krämer, 2000; Cheung *et al*., 2003; Nagarajavel *et al*., 2007; Romeo *et al*., 2007; Rosenthal *et al*., 2008; Altendorf *et al*., 2009; Krämer, 2010). Information on osmotically controlled gene expression is available for only a few of the functionally characterized BCCT carriers (Table 1). For instance, osmotic induction of *betP* transcription is governed by the two-component MtrAB regulatory system of *C. glutamicum* (Möker *et al*., 2004; 2007). Expression of the *B. subtilis opuD* gene is partially dependent on the alternative transcription factor SigB (F. Spiegelhalter and E. Bremer, unpubl. data), the master regulator of the general stress regulon. SigB also controls the osmotic induction of *ectT* from *Virgibacillus pantothenticus* (A.U. Kuhlmann and E. Bremer, unpubl. data). The substrates of osmotically induced BCCT carriers typically do not trigger gene expression of their corresponding structural genes, with the exception of the choline transporters BetT from *E. coli* (Lamark *et al*., 1996) and CudT from *Staphylococcus xylosus* (Rosenstein *et al*., 1999), where unrelated choline-responsive repressor proteins (BetI and CudC) have been identified. Osmoregulation and simultaneous substrate induction of *betT* and *cudT* transcription is of physiological relevance because the function of choline as an osmoprotectant is entirely dependent on its conversion into glycine betaine.

It is increasingly recognized that compatible solutes not only function as microbial osmoprotectants, but serve other stress protective functions as well (Yancey, 2005; Empadinhas and da Costa, 2008). In particular, a compatible-solute-mediated temperature stress protection both at the upper and lower boundaries of growth has been observed in various microbial species. BCCT carriers participate in these processes in several microorganisms. Examples are the BetT-mediated uptake of choline in *E. coli* (Caldas *et al*., 1999) and the OpuD-mediated uptake of glycine betaine in *B. subtilis* as heat stress protectants (Holtmann and Bremer, 2004), the chill stress protection by glycine betaine via BetL-mediated import *in Listeria monocytogenes* (Angelidis and Smith, 2003; Sheehan *et al*., 2006) and OpuD in *B. subtilis* (T. Hoffmann and E. Bremer, unpubl. data) and the EctT-mediated uptake of ectoine/hydroxyectoine in *V. pantothenticus* (A.U. Kuhlmann and E. Bremer, unpubl. data).

The BetP transporter from *C. glutamicum* and other BCCT carriers: structure, function and regulation

Function of BetP from C. glutamicum *in response to osmotic stress*

In comparison to other bacteria such as *E. coli* (Altendorf *et al*., 2009) or *B. subtilis* (Bremer, 2002), which employ a combination of primary transporters (the ABC transporters) and secondary transporters, uptake of compatible solutes in *C. glutamicum* is mediated entirely by osmoregulated secondary transporters (Peter *et al*., 1998b). Three of them, BetP, EctP and LcoP, are members of the BCCT family. These transporters vary in substrate specificity, affinity, and transcriptional regulation, but all respond to hyperosmotic stress by regulating the level of transport activity (Krämer, 2009). BetP is by far the best described, both with respect to transport and regulation. BetP is specific for glycine betaine and couples its uptake to the electrochemical Na^+ potential (smf) by co-transport with two Na^+ ions, leading to extremely high accumulation ratios up to 10^6 and internal concentrations above 1 mol l^{-1} (Farwick *et al*., 1995; Peter *et al*., 1996).

The activity of BetP is close to zero in the absence of hyperosmotic stress. Upon osmotic challenge, BetP starts taking up glycine betaine in less than a second (Peter *et al*., 1998a). When transport activity is plotted against the extent of osmotic stress, a steep sigmoidal increase in activity is observed until an optimum is reached; in intact *C. glutamicum* cells this occurs around 1.3 osmol kg^{-1} (Fig. 3). Consequently, besides its catalytic activity (Na^+-coupled glycine betaine uptake), BetP acts both as an osmosensor and an osmoregulator by responding to osmotic stress with instant regulation of its transport activity. This fact was documented both by heterologous expression of the *betP* gene in *E. coli* (Peter *et al*., 1996), and by reconstitution of the purified BetP protein in proteoliposomes (Rübenhagen *et al*., 2000). In these two artificial membrane surroundings, the BetP transporter from *C. glutamicum* retained all its functions, indicating that no other proteinaceous activating factors are involved in controlling BetP activity. The differences in the activa-

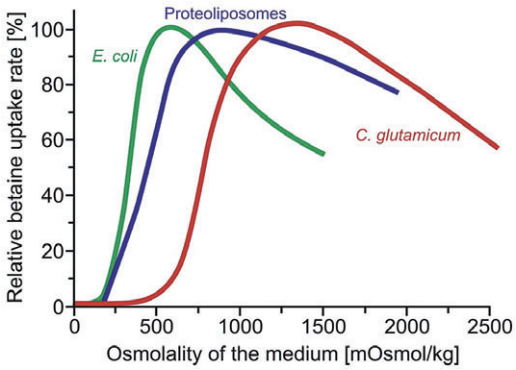

Fig. 3. Activation profile of the BetP transporter embedded in different membranes. The rate of glycine betaine uptake was measured as a function of the osmolarity of the surrounding medium, either in the native membrane of *C. glutamicum* (red line), after heterologous expression in *E. coli* (green line), or after isolation, purification and reconstitution into proteoliposomes (blue line). The maximum activity measured was set to 100%, the absolute values are in the range of 30–80 μmol (min·mg dw)$^{-1}$ for cells and about 1000–2000 μmol (min·mg protein)$^{-1}$ for proteoliposomes.

tion pattern, observed in Fig. 3, can be explained by a significantly different share of negatively charged phospholipids in the membranes of the two different organisms (Schiller *et al*., 2006).

Activation of BetP is likely modulated through transitions between at least two different functional and conformational states: inactive or active. Hyperosmotic stress will shift the balance towards the active state, while hypoosmotic stress will favour the inactive conformation of BetP. Thus, the function of BetP can be described by a kinetic scheme (Fig. 4) comprising two functional cycles: (i) The catalytic cycle of glycine betaine transport corresponds to that of a secondary transporter. It includes a conformational change between outward and inward facing carrier molecules (Jardetzky, 1966) and a cycle between substrate binding and release. The turnover in this cycle depends on both glycine betaine availability and the energetic driving force. (ii) The balance between the two-state regulatory cycles of BetP depends on certain stimuli; some of them are related to osmotic stress. An assumption inherent in the regulatory cycle of BetP as depicted in Fig. 4 is that the occluded states of the transporter have been chosen as the points where the two cycles connect, but the mechanistically correct connection point is not known so far. During both the catalytic and the regulatory cycle, BetP passes through several functional states and, consequently, must adopt different conformations.

X-ray structures of BetP and CaiT: structural models for members of the BCCT family

BCCT carriers involved in osmotic stress response catalyse Na^+ or H^+ symport, during which the smf or pmf drives the conformational change from an outward-facing to an inward-facing state according to the alternating access mechanism of secondary transporters (Jardetzky, 1966) (Fig. 4). Structural data are required in order to understand the mechanisms of ion-coupled osmolyte symport by BCCT carriers. Crystal structures of two members of this transporter family are available to date: the structure of BetP from *C. glutamicum*, which was solved by X-ray crystallography to 3.35 Å resolution in complex with its substrate glycine betaine (Ressl *et al*., 2009) (PDB entry 2WIT), a 3.15 Å structure of CaiT from *E. coli* in complex with four L-carnitine molecules (Tang *et al*., 2010) (PDB entry 3HFX), a 3.5 Å structure of CaiT from *E. coli* in complex with two γ-butyrobetaine molecules (PDB code: 2WSX), and an apo structure of CaiT (PDB entry 2WSW) from *P. mirabilis* at 2.3 Å (Schulze *et al*., 2010). Although CaiT shares the least sequence identity to BetP of all functionally characterized BCCT carriers listed in Table 1, the crystal structures of BetP and CaiT superimpose quite well with a root mean square deviation of ~2.5 Å. Both the purified BetP and CaiT proteins form trimers (Jung *et al*.,

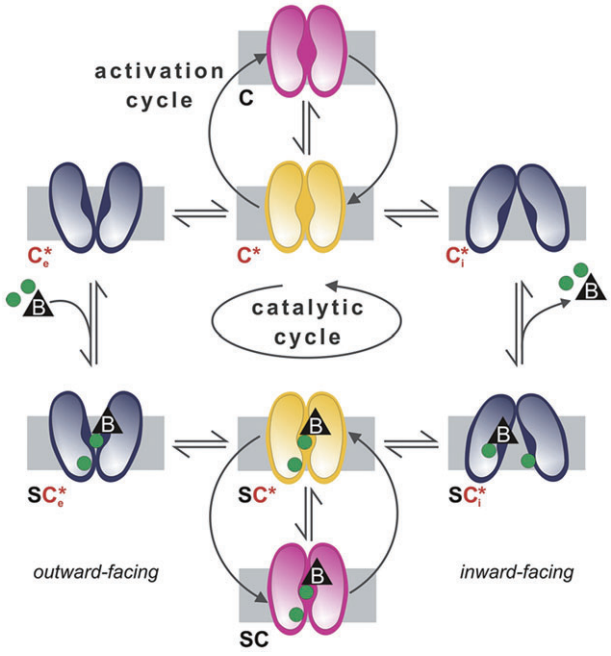

Fig. 4. Schematic representation of the catalytic and the activation (regulatory) cycle of the BetP transporter. In the activation cycle, BetP (C = carrier) occurs either in the active (C*, blue and yellow colour) or the inactive state (C, red colour). In the catalytic cycle, BetP exposes its glycine betaine substrate binding site either to the periplasmic space (C^*_e, carrier protein symbol open to the top) or to the cytoplasmic space (C^*_i, carrier protein symbol open to the bottom). Alternatively, the substrate binding site is occluded (yellow colour) from the surroundings. Substrate (S) denotes one molecule of glycine betaine (B; black triangel) together with two Na^+ ions (green circles). In the course of one round of the catalytic cycle of the BetP carrier, one molecule of glycine betaine and two Na^+ ions are translocated across the plasma membrane.

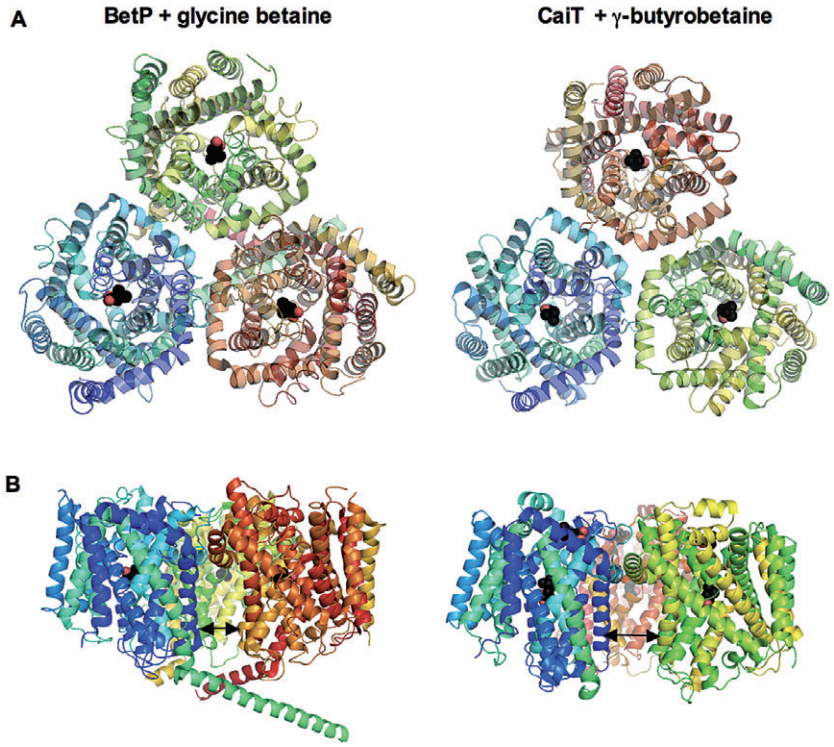

Fig. 5. Crystal structure of BetP from *C. glutamicum* and CaiT from *E. coli* in complex with their ligands.
A. Periplasmic view on the BetP trimer (resolved to a resolution of 3.35 Å) in spiral presentation and on the CaiT trimer (resolved to a resolution of 3.5 Å). Individual subunits are coloured in blue, red and green.
B. Side view on the BetP and CaiT trimers. The C-terminal domain of subunit 1 from the BetP trimer assembly is shown to protrude 35 Å into the cytoplasm.

2002; Ziegler *et al.*, 2004; Vinothkumar *et al.*, 2006) and both proteins also crystallize as trimers (Fig. 5). Each subunit consists of 12 TM α-helices and contains a curved amphipathic α-helix (helix 7) that runs parallel to the plane of the cytoplasmic membrane and a long bend TM helix (TM2), which both provide a hydrophobic scaffold surrounding the centre of the transporter (Fig. 6). Helix 7 is located at the trimer centre of both the BetP and CaiT transporters, close to the periplasmic membrane surface, where it mediates contacts between all three subunits within the trimer at the periplasmic side.

A feature that distinguishes BetP from CaiT is a α-helical C-terminal domain protruding into the cytoplasm (Fig. 5B). In BetP, the C-terminal domain on the cytoplasmic side contributes to trimer contacts and as a result, a slightly different subunit arrangement within the trimers of the BetP and CaiT crystal structures are observed (Fig. 5B). In comparison to BetP, each subunit within the

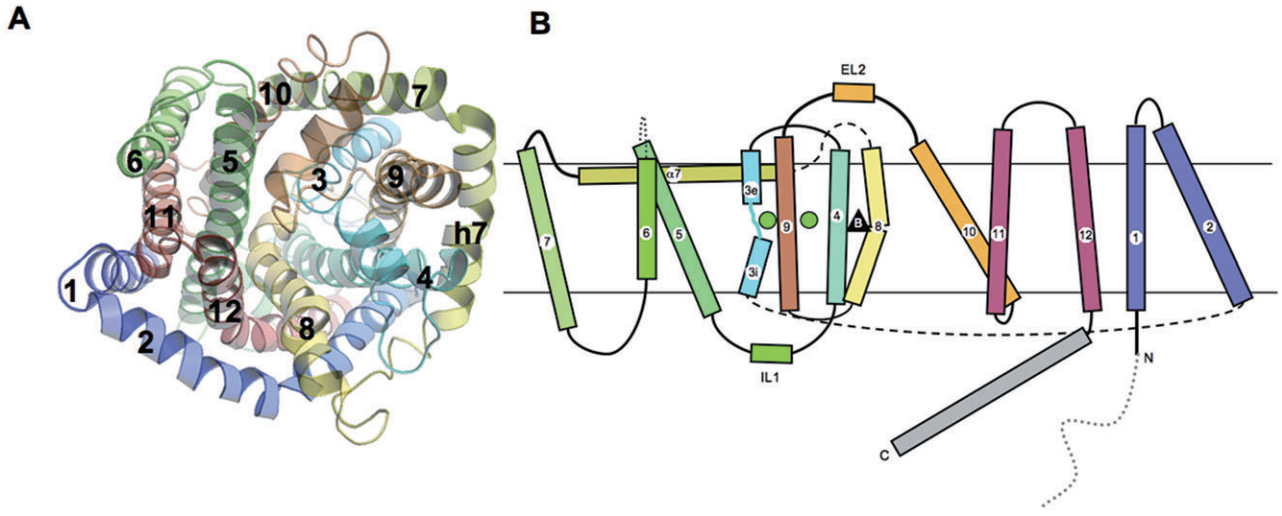

Fig. 6. Toplogical organization of BetP within the plain of the cytoplasmic membrane. (A) Top view on a BetP subunit with a colouring of the TM helices assigned according to BetP's topology (B) showing the organization of the two structural repeats.

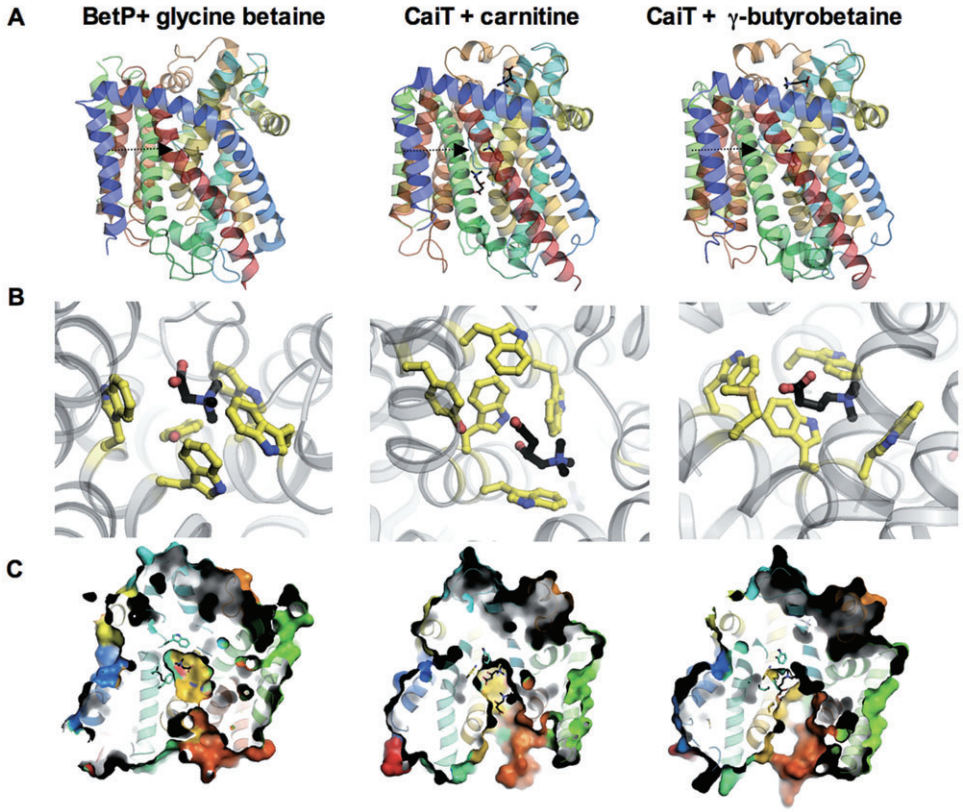

Fig. 7. Structural features and ligand binding sites of BetP and CaiT.
A. Side view on the *C. glutamicum* BetP structure in complex with one molecule of glycine betaine; the CaiT structure from *E. coli* in complex with four L-carnitine molecules and the CaiT structure from *P. mirabilis* in complex with two γ-butyrobetaine molecules.
B. Structural features of the central ligand binding site in BetP and CaiT. The aromatic ligand-binding boxes co-ordinating the substrate glycine betaine, L-carnitine or γ-butyrobetaine are highlighted.
C. Cross-section through the BetP and CaiT structures revealing an occluded state for BetP and an inward-facing state for CaiT respectively.

trimer assembly is tilted somewhat resulting in a larger cavity in the trimer centre of CaiT (Fig. 5B, black arrow). TM3, TM4, TM8 and TM9 form an iris-shaped 4-helix bundle close to the centre of both the BetP and CaiT subunit, with aromatic side-chains in TM4 and TM8 contributing to the substrate binding site (Fig. 7B).

The 3.15 Å crystal structure of CaiT from *E. coli* reveals four L-carnitine molecules (Fig. 7A) most likely because the protein was crystallized with an excess of L-carnitine. The primary binding site at the centre of the protein and the secondary substrate binding site at the bottom of the intracellular vestibule have been confirmed by site-directed mutagenesis studies (Tang *et al.*, 2010), while the two other L-carnitine molecules might be associated with the crystallized CaiT protein for structural reasons. The ligand binding site in one of the *E. coli* CaiT structures contains a bound γ-butyrobetaine (Schulze *et al.*, 2010), the substrate that is exported from the *E. coli* cell after metabolism of L-carnitine under anerobic growth conditions (Eichler *et al.*, 1994; Jung *et al.*, 2002). As observed in the CaiT structure in complex with L-carnitine, the bulky headgroup of γ-butyrobetaine is co-ordinated by cation-pi interactions and van-der-Waals contacts. In contrast, the central binding site of the *P. mirabilis* CaiT structure does not contain any bound substrate. A second γ-butyrobetaine molecule is bound in the extracellular cavity of the *E. coli* CaiT protein (Fig. 7A). Instead of γ-butyrobetaine, two ordered water molecules occupy the corresponding position in the *P. mirabilis* CaiT protein, most likely because access of the substrate is blocked by crystal contacts. The functional role of this putative second ligand binding site is unclear. The different CaiT crystal structures revealed that two of the L-carnitine binding sites are identical to those containing γ-butyrobetaine, while the other two L-carnitine sites most likely contain non-specifically bound substrate (Schulze *et al.*, 2010; Tang *et al.*, 2010).

The central binding site (Fig. 7B) of L-carnitine and γ-butyrobetaine in CaiT corresponds to the glycine betaine binding site present in BetP (Ressl *et al.*, 2009). However, the bound L-carnitine and γ-butyrobetaine molecules in the CaiT structures are accessible from the

cytoplasm, while in the BetP structure, the access to the bound glycine betaine molecule is occluded from both sides of the membrane (Fig. 7C). In TM4 and TM8 of BCCT carriers, highly conserved aromatic residues, including those that are part of the BCCT signature sequence (Kappes *et al*., 1996; Saier, 2000), stabilize the 4 TM helix bundle in the CaiT and BetP subunits. The amino acid sequence of TM3 in the BetP and CaiT proteins is not conserved; however, both crystal structures revealed that this TM region has a locally unwound segment approximately halfway across the membrane (Fig. 7A). In BetP, TM3 harbours one of the Na^+ ion binding sites. This sodium ion contributes to the co-ordination of the carboxyl group of glycine betaine (Fig. 2) and, thereby, stabilizes the ligand within in its binding site (Ressl *et al*., 2009), while the second sodium ion stabilizes the unwound segment of TM3. Unlike BetP, CaiT is a Na^+-independent transporter and the functional role of the locally unwound segment in TM3 is therefore not immediately obvious. However, two residues present in TM3 of CaiT are involved in interactions with the L-carnitine molecule trapped in both the primary and secondary binding site. Interestingly, an Arg residue in TM7 in CaiT occupies the position of the second Na^+ binding site present in BetP. Similar to Na^+, the positive charge of the Arg amino acid side-chain stabilizes the unwound helix in TM3. The co-ordination of the carboxyl group of the γ-butyrobetaine ligand is maintained in CaiT by the sulphur of a Met residue that is highly conserved in CaiT-related proteins, but it is absent in BetP-related proteins.

The crystal structures of BetP (Ressl *et al*., 2009) and of CaiT (Schulze *et al*., 2010; Tang *et al*., 2010) share a strikingly similar architecture with that of other Na^+-coupled symporters like LeuT (Yamashita *et al*., 2005; Singh *et al*., 2007; 2008; Zhou, 2007), vSglT (Faham, 2008) and Mhp1 (Weyand, 2008), with H^+-coupled symporters like ApcT (Shaffer *et al*., 2009) and antiporters like AdiC (Gao *et al.,* 2010), despite the fact that they are not related at the amino acid sequence level. Each of these transporters possesses a core domain consisting of 10 TM helices. Hydropathy profile alignments (Lolkema and Slotboom, 2008) confirm the structural similarity between transporters of the neurotransmitter : sodium : symporter family (LeuT), the sodium : solute : symporter family (vSglT), and the aminoacid : polyamine : organocation family (ApcT) (Gouaux, 2009; Krishnamurthy *et al*., 2009; Shaffer *et al*., 2009). These 10 C-terminal TMs (corresponding to TM3-12 in BetP) contain an inverted repeat of two 5 TM helixes each. Repeat 1 (TM3-TM7 in BetP) is a structural homologue of repeat 2, and they are related by pseudo twofold symmetry axis running parallel to the membrane (Fig. 6B). Substrate and co-substrate binding sites are all located in the interface between the two intertwined repeats. A superposition of the core structures of this group of transporters reveals a root mean square deviation of 3.8–4.5 Å, indicating not only a high degree of structural conservation with respect to the overall fold but also to the position of the substrate and co-substrate binding sites (Caplan *et al*., 2008; Noskov and Roux, 2008; Ressl *et al*., 2009). The common core structure, the similar location of the substrates in these transporters and the fact that the crystal structures reflect distinct conformations were recently used to propose a common molecular mechanism for Na^+- and H^+-coupled symport and antiport (Abramson and Wright, 2009; Gouaux, 2009; Krishnamurthy *et al*., 2009; Shaffer *et al*., 2009).

The Na^+-coupled glycine betaine symporter BetP and the L-carnitine/γ-butyrobetaine antiporter CaiT represent different subgroups within the BCCT carriers (Table 1). However, the striking similarities of their crystal structures provide now a solid basis for formulating unifying concepts that describe functional mechanisms operating within the BCCT family. In our comparisons of a large number of BCCT carriers, we noted a high amino acid sequence conservation of those residues that are involved in coupling and substrate binding within the BCCT family. The degree of sequence identity in TM3, TM4 and TM8 is very high within the BCCT family. However, there are two distinct pattern of conservation: (i) residues involved in ion-coupling or counter-substrate coupling and (ii) residues involved in binding of the fully methylated head group of various compatible solutes (Figs 2 and 7B). Therefore, the structures of BetP and CaiT probably can be used to explain transport of other methylated ammonium or sulphur compounds, such as choline and DMSP, and permit structure-guided speculations on the transport mechanism of non-quaternary ammonium substrates of BCCT carriers, such as ectoine and hydroxyectoine (Fig. 2). Thus, the BetP and CaiT structures allow us to search for key residues that are likely to be involved in functionally relevant conformational changes in BCCT carriers.

Substrate binding: what determines substrate specificity and coupling mode in the BCCT family?

In general, it is not possible to predict with confidence the substrate profile of a given BCCT carrier solely by inspecting and aligning amino acid sequences. However, the BetP and CaiT structures reveal a striking pattern in substrate binding that allows one, at least to some extent, to predict the substrate profile of other BCCT carriers based on sequence alignments. Glycine betaine transporters of the BCCT family show a substantial degree of sequence relatedness (between 48% and 40%) in the 10 TM transporter core (excluding the N-terminal and C-terminal extensions). In contrast, the amino acid sequence relatedness of the 10 TM core falls below 40% for ectoine and

choline transporters and even lower (below 30%) for L-carnitine/γ-butyrobetaine antiporters.

The BetP crystal structure revealed the architecture of the binding site for its ligand glycine betaine and the functional importance of key residues within this binding site were confirmed by site-directed mutagenesis studies (Ressl *et al*., 2009). The architecture of the ligand binding site present in BetP is nearly identical with the primary ligand binding site for L-carnitine and γ-butyrobetaine in CaiT (Schulze *et al*., 2010; Tang *et al*., 2010). Hence, the crystal structures of BetP and CaiT revealed for the first time the molecular determinants for substrate binding of trimethylammonium compounds by members of the BCCT carriers. In particular, the indole groups of three tryptophane residues, one positioned in TM4 and two positioned in TM8, are arranged in the form of an aromatic cage in which the fully methylated head groups of glycine betaine, L-carnitine and γ-butyrobetaine (Fig. 2) are stabilized via cation-pi interactions. The carboxyl groups of these ligands protrude out from this aromatic cage and are co-ordinated via hydrogen bonding interactions within the ligand binding site (Fig. 7B). The co-ordination of the glycine betaine, L-carnitine and γ-butyrobetaine substrates within the BetP and CaiT transporters show striking similarities to that found in the ligand-binding proteins (ProX) of the glycine betaine ABC-type uptake system from *E. coli* (Schiefner *et al*., 2004a) and the hyperthermophilic archaeon *Archeoglobus fulgidus* (Schiefner *et al*., 2004b). The ProX proteins are soluble and function in the context of ABC transporters and are thus not evolutionarily closely related to the integral membrane proteins BetP and CaiT. Hence, nature has used common design principles to achieve the selective and high-affinity binding of this group of compatible solutes by proteins, compounds that are typically preferentially excluded from the hydration shell of the protein backbone (Arakawa and Timasheff, 1985; Bolen and Baskakov, 2001; Street *et al*., 2006; 2010; Rösgen, 2007; Bolen and Rose, 2008). The crystal structures of ligand-binding proteins from ABC- and TRAP-transport systems revealed that the binding of compatible solutes in substrate-selective binding sites present in transporter proteins seems to require a certain number of aromatic residues for substrate co-ordination via cation-pi and van der Waals interactions in order to provide a surface that minimally repels osmolytes (Schiefner *et al*., 2004a; Horn *et al*., 2006; Hanekop *et al*., 2007; Kuhlmann *et al*., 2008; Smits *et al*., 2008; Capp *et al*., 2009; Lecher *et al*., 2009; Ressl *et al*., 2009; Schulze *et al*., 2010; Tang *et al*., 2010; Wolters *et al*., 2010). Several crystal structures of binding proteins from ABC- or TRAP-type transport systems in complex with compatible solutes used by BCCT carriers (Table 1) have been reported (Schiefner *et al*., 2004a,b Horn *et al*., 2006; Hanekop *et al*., 2007; Kuhlmann *et al*., 2008; Oswald *et al*., 2008; Smits *et al*., 2008; Lecher *et al*., 2009; Wolters *et al*., 2010). Hence, the co-ordination of the substrates in these soluble proteins might give valuable clues to substrate binding site(s) present in BCCT carriers for which no crystal structure is yet available (e.g. transporters for ectoines and DMSP).

Most of the BCCT carriers show a high degree of conservation of the Trp residues in TM4 and TM8 that co-ordinate the trimethlyammonium ligands in BetP and CaiT (Ressl *et al*., 2009; Schulze *et al*., 2010; Tang *et al*., 2010), but these residues cannot be identified in ectoine/hydroxyectoine transporters of the BCCT family. From these patterns of conservation, we conclude that the co-ordination of ectoine or hydroxyectoine, which lack a quaternary ammonium group (Fig. 2), is different from that of glycine betaine, γ-butyrobetaine or L-carnitine, while co-ordination of choline is likely to be similar. This latter suggestion is supported by the molecular determinants governing choline binding by the periplasmic ligand binding ChoX protein from *Sinorhizobium meliloti* that also archives the binding of the methylated head group of choline through cation-pi interactions in an aromatic ligand-binding cage (Oswald *et al*., 2008).

Like glycine betaine, ectoine and hydroxyectoine carry a negatively charged carboxylate (Fig. 2). Hence, it is tempting to speculate that a sodium ion might interact directly with the carboxylate of ectoine and hydroxyectoine in a similar way to that found in BetP (Ressl *et al*., 2009). The co-ordinating Na^+ ion in the BetP crystal structure is located halfway across TM3, close to an unwound, glycine-rich segment that separates TM3 into two segments (Fig. 7B). This flexible segment in BetP contains a GMGIG motif that is conserved in glycine betaine-specific transporters of the BCCT family. TM3 in the Na^+-independent substrate : product antiporter CaiT shows now sequence homology in this region, while ectoine-specific BCCT carriers show sequence modifications at the corresponding position. Substrate co-ordination by residues in TM3 might also be crucial for proton-coupled BetT-like proteins of the BCCT family. The chemical nature of glycine betaine and choline differs with respect to their carboxyl and alcohol groups (Fig. 2). While salt bridges to the substrate are maintained in Na^+-coupled transporters by the coupling ion, this might not be the case for choline, which carries a positive charge (Fig. 2). However, the choline transporter BetT from *E. coli* comprises a charged residue in the flexible segment in TM3. The presence of a side-chain capable of reversible protonation during the transport cycle might be a prerequisite for the H^+-coupled transport, since such an arrangement was also observed in the H^+-coupled amino acid transporter ApcT from *Methanocaldococcus jannaschii* (Shaffer *et al*., 2009).

There might be substrates for BCCT carriers that are different from those compiled in Fig. 2. Some putative

BCCT carriers harbour conserved, but significantly different motifs of the glycine betaine, choline, ectoine or carnitine transporters of the BCCT family. This functionally uncharacterized group of BCCT carriers is predominantly found in members of the *Firmicutes*, and they also form the major group of BCCT carriers present in *Synechococcus*, a subclass of cyanobacteria. Depending on the degree of their salt stress resistance these microorganisms synthesize sucrose, trehalose, glucosylglycerol and glycine betaine under salt stress (Hagemann, 2010). Some marine *γ-Proteobacteria* possess BCCT carriers with a very low degree of sequence homology to BetP (approximately 20%) and, most strikingly, they lack TM1. Accordingly, these transporters have a predicted transmembrane topology that is different from that of BetP. These considerations suggest that it might be rewarding to study these so-far uncharacterized BCCT carriers with respect to their potential substrates, their transport activity profiles and topological organization within the cytoplasmic membrane.

The oligomeric state of BCCT carriers and their terminal domains

BetP and CaiT are both trimeric in detergent solution (Ziegler *et al*., 2004; Vinothkumar *et al*., 2006), as well as in the membrane when analysed by 2D electron crystallography, and in the 3D crystal (Ressl *et al*., 2009; Schulze *et al*., 2010; Tang *et al*., 2010; C.-J. Tsai *et al*., submitted). The observed trimer formation is a consequence of the unique structural elements characterizing the fold of the BCCT carriers, which are the amphiphatic helix 7 and the long bend TM2. They provide a hydrophobic interface, which forces a triangular subunit arrangement for stability reasons. In fact, BetP and CaiT have proven to be very stable both in detergent solution and when reconstituted back into the membrane (Tsai *et al*., 2007). It is thus tempting to speculate that trimer-formation is a general feature of the BCCT carriers with functional impact for transport and/or regulation. We have analysed the differences in trimer contacts in the osmotically regulated BetP and the osmotically non-regulated CaiT proteins to validate this assumption. The three subunits within the CaiT and BetP trimer form a central conical cavity lined by aromatic side-chains from TM2, TM3 and h7 (Fig. 5A). The hydrophobic cavity in the trimer centre is most likely filled with lipid as observed for the crystallized BetP protein (Ressl *et al*., 2009). Residues contributing to the formation of this cavity are highly conserved in members of the BCCT carriers. On the periplasmic side, the amphipathic helix 7 is the key player in maintaining trimer contacts (Fig. 6). Helix h7 in BetP and CaiT is rich in Leu and Asp residues and shows characteristics of a non-specific lipid interaction site that stabilizes the trimer assembly. This helix represents a structurally distinct feature of the BCCT carriers since it is not present in any other transporter sharing the same overall inverted structural repeat (e.g. LeuT). In BetP and CaiT, helix h7 separates the two structural repeats from each other and therefore cannot be attributed to either of the repeat parts of the transpiorter proteins. Contacts involve mainly hydrophobic residues in helix h7, TM2, TM3 and loop7 of adjacent subunits. Residues participating in trimer contact via helix h7 are not entirely conserved within the BCCT carriers. CaiT shares only 17% sequence identity in this region to the BetP protein. However, the two structurally important polar interactions observed in BetP are also found in CaiT and are predicted to occur in eight other BCCT carriers listed in Table 1. In CaiT and nine other BCCT carriers, additional subunit–subunit contacts are likely to be promoted by ionic interactions, resulting in a quite rigid trimer assembly; in contrast, in BetP they are formed by more flexible hydrophobic interactions. However, only $BetP_{Cg}$ and $OpuD_{Vc}$ (Table 1) seem to have exclusively hydrophobic interactions in their trimer contact regions as found in BetP. The majority of BCCT carriers show (i) a conserved length of h7 and (ii) the potential for both hydrophobic and ionic interactions via helix h7. Both parameters favour a triangular interface, while a dimer or a tetramer formation is not possible. Therefore, we can safely assume that the majority of BCCT carriers form trimers in the plane of the cytoplasmic membrane. It seems that BetP and CaiT represent two different structural solutions within the BCCT carriers to foster trimerization.

It is an intriguing question whether the flexible trimer interactions observed in BetP in comparison to those found in CaiT can be related to BetPs osmoregulatory abilities. Subunit interactions within the BetP trimer not only involve helix h7 but also involve the C-terminal helix of each BetP subunit within the trimer assembly; this C-terminal extension interacts with loop 2 and the C-termini of the other two BetP subunits. Only when the subunits are arranged in a trimer, individual C-terminal domains of BetP would not hinder each other sterically. The C-terminal helix of BetP is not found in CaiT (Fig. 5). The additional trimer contact formed by the C-terminal helix of BetP within the BetP trimeric structure is of particular interest because biochemical studies have provided compelling evidence for the involvement of this domain in osmosensing and regulation (Ott *et al*., 2008). The C-terminal domain of BetP contains several positively charged residues clustered at the N-terminal part of the C-terminal domain (cluster 1: R554, R558, R565, R567 and R568) and near the end of the C-terminal domain (cluster 2: R576, K581, R582–584, R586 and K587). It lacks Gly and/or Pro residues, except close to its very end, and thus forms a straight α-helix (Figs 5B and 6B). Biochemical data revealed the functional importance of the

conformation and position of the first charge cluster within the C-terminal domain of BetP (Ott *et al*., 2008; Ressl *et al*., 2009). In the context of trimer contacts in BetP, arginines in the first cluster form salt bridges to a glutamate separating both clusters in the C-terminal domain of the adjacent subunit. In the 22 functionally characterized microbial BCCT carriers (Table 1), the C-terminal domains do not have a conserved sequence, but three different motifs can be observed: (i) BetP-type C-terminal domains feature two clusters with opposite charges positioned several residues apart from each other, but lack flexible stretches of Gly and/or Pro residues. (ii) CaiT-type C-terminal domains have, at most, very short charged clusters. Consequently, the BetP-type C-terminal domains present in $BetP_{Cg}$, $BetT_{Ah}$, CudT, $BetH_{Ht}$, $OpuD_{Bs}$, EctM, ButA and EctP (Table 1) are likely to contribute to trimer contacts, while CaiT-type C-terminal domains present in BetM and DddT (Table 1) do not do so. (iii) There is a third type of C-terminal domain in members of the BCCT carriers. It consists of charged clusters but is also rich in Gly and/or Pro residues, suggesting that these C-terminal domains are not entirely α-helical. This type of C-terminal domain is often very long (> 70 aa) and is found in the $BetT_{Ec}$, $BetT_{Ps}$, $BetP_{Hi}$, BetS and BetU transporters (Table 1). For several members of the BCCT carriers, namely BetL, $BetP_{Vp}$, $OpuD_{Vc}$ and LcoP (Table 1), the assignment to the three types of C-terminal domains is not a trivial task, since they all comprise charged amino acid clusters together with flexible stretches of glycine and/or proline residues. A BetP-like helix h7 and a BetP-like C-terminal domain are found together only in the glycine betaine transporter ButA from *Tetragenococcus halophila* (Table 1) (Baliarda *et al*., 2003).

Interactions of C-terminal domains with the cytoplasmic side of adjacent subunits are one of the main differences between the BetP (Ressl *et al*., 2009) and the CaiT structures (Schulze *et al*., 2010; Tang *et al*., 2010). The C-terminal interactions are not symmetrical in the BetP trimer, resulting in a break in non-crystallographic threefold symmetry. A break in symmetry was also observed for BetP embedded in the membrane in 2D crystals (Ziegler *et al*., 2004), while the CaiT trimer exhibits a perfect threefold symmetry. Unfortunately, electron crystallographic data of CaiT are not available yet at the required resolution, but with respect to trimer formation it is very likely that CaiT is also a symmetric trimer in the membrane. In contrast, each subunit in the BetP trimer adopts a distinct conformation (C.-J. Tsai *et al*., submitted). The different conformations of each subunit in membrane-embedded BetP, the structural flexibility of the C-terminal domains and of the BetP trimer suggest a role of trimerization far beyond protein stabilization in BetP. Together with the fact that C-terminal domains act as osmoregulators, the observed C-terminal interactions point towards a functional role in transport activity regulation in BetP (Krämer, 2009). A possible scenario would be that the subunits communicate their conformational state via the trimer interface to synchronize their transport activity. Thereby, the BetP glycine betaine transporter would take advantage of the trimeric state in a very elegant fashion.

Sensing and signal transduction by BetP at the biochemical level

A considerable number of the BCCT carriers listed in Table 1 respond to osmotic stress but this issue has only been studied in detail for a few of these transporters. In view of the multiplicity of potential physical stimuli directly or indirectly related to osmotic stress (Wood, 1999; 2006; Bremer and Krämer, 2000; Poolman *et al*., 2004; Altendorf *et al*., 2009; Krämer, 2009), the analysis of the sensory input into a transporter protein is an experimentally difficult task. For BetP, the situation was simplified by the fact that the reconstituted protein in proteoliposomes was fully capable of responding to osmotic stress (Rübenhagen *et al*., 2000). Consequently, individual physical stimuli could be tested separately and their relevance for carrier activation could be carefully evaluated.

The physical stimuli found to be relevant for the osmotic control of BetP activity can be divided into two classes: (i) an increase in K^+ (and Rb^+ or Cs^+) triggers BetP activity but only when applied at the cytoplasmic side (Rübenhagen *et al*., 2001). The observed specificity with respect to the activating agent practically excludes the unspecific effect of a change in the degree of BetP hydration as a stimulus. An unspecific influence of osmolytes and macromolecules has been proposed as a stimulus for osmotic control of the MFS-type transporter ProP from *E. coli* (Culham *et al*., 2003). (ii) BetP activity is also affected by stimuli originating from the membrane. A modulating effect of local anaesthetics inserted into the membrane affects BetP-mediated glycine betaine transport (Steger *et al*., 2004). This is not an unspecific effect, since these types of compounds did not affect the BCCT carrier EctP from *C. glutamicum*. It was also observed that the surface charge density of the surrounding membrane strongly modulates BetP activity; the concentration of K^+ necessary for BetP activation increased in parallel to the content of negatively charged lipids (Schiller *et al*., 2006). In addition to osmotic stress, BetP was also activated by chill stress (Özcan *et al*., 2005). Since this effect was independent of changes in K^+ content the relevant stimulus should originate from changes in the physical state of the membrane. Finally, membranes with changed lipid composition but unchanged surface charge density exhibit altered regulation of the BetP transporter (Özcan *et al*., 2007). Taken together, signal input leading to stimulation

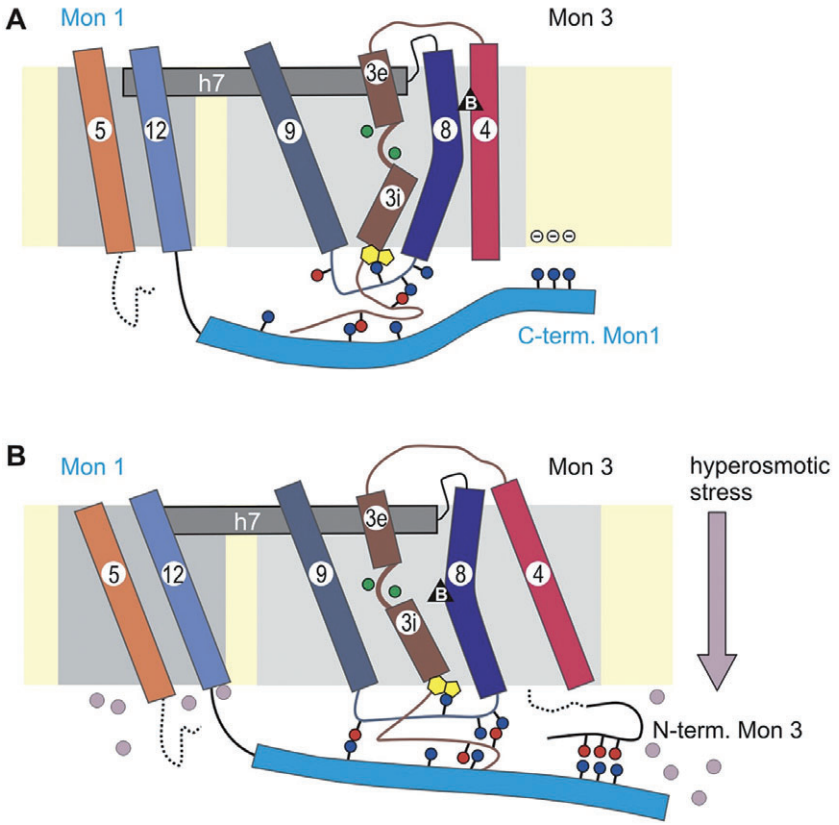

Fig. 8. Osmoregulatory interactions within the BetP trimer. Spatial interaction of the C-terminal domain of one subunit (monomer 1) of BetP with charged residues in loop 2 and loop 8 of the adjacent BetP subunit (monomer 3), which is suggested to be of significance for activity regulatory.
A. Under low external osmolarity and/or absence of K^+ ions (inactive conditions) the BetP carrier is locked in a resting conformation with the bound two Na^+ ions (green circles) and glycine betaine (B; black triangle) occluded from the cytoplasmic side.
B. Under hyperosmotic conditions and increasing concentrations of K^+ ions (grey spheres) in the cytoplasm and stimuli perceived via the membrane, the BetP carrier initiates a orientation/conformation change of its C-terminal domain which alters the interaction network and allows a conformational change of the TM helices of the inverted repeats. As a consequence, glycine betaine can be released into the cytoplasm. The role of the N-terminal domain of BetP (monomer 3) in this interaction switching is currently not firmly understood. It is suggested that the negative charge clusters in BetP (red spheres) interact with the positive charge clusters (blue spheres) present in the C-terminal domain of BetP (picture kindly provided by Susanne Ressl).

of BetP transporter activity seems to be of two types: a rise in the intracellular K^+ concentration that follows an osmotic up-shift and a stimulus originating from the membrane into which the BetP transporter is embedded.

Mutational studies of BetP have provided substantial information on the contribution of selected domains with respect to its transport function and regulation of its activity by high osmolarity. A stepwise truncation of the C-terminal domain of BetP showed that the C-terminal region containing the second charge cluster could be deleted without significant impact on transporter function. Further truncation of the C-terminal domain by 25 amino acid residues or more, including the first charge cluster, led to BetP transporter derivatives with decreased but constitutive activity (Peter *et al.*, 1998a; Schiller *et al.*, 2004). Full removal of the C-terminal domain rendered BetP inactive. Interestingly, activation of BetP activity at low temperature, probably by a stimulus derived from the surrounding membrane, was not influenced by a truncation of the C-terminal domain (Özcan *et al.*, 2005). The functional significance of the C-terminal domain of BetP was further studied by amino acid scanning mutagenesis (amino acid replacements with Ala or Pro residues) (Ott *et al.*, 2008). These experiments led to two important conclusions: (i) a particular region in the middle of the C-terminal domain of BetP was found to be critical for sensory function with respect to its conformational properties rather than its charge distribution, and (ii) interaction of the C-terminal domain with particular loops at the cytoplasmic face of BetP and with the N-terminal domain was detected (Ott *et al.*, 2008). Consequently, both terminal domains, and in particular the C-terminus, are critically involved in the activation of BetP by osmotic stress. BetT-like choline transporter proteins have C-terminal domains that are substantially longer than that present in BetP (Table 1). In both *E. coli* and *P. syringae*, BetT proteins with truncated C-terminal domains gradually lose their capacity for osmotic activation, indicating that these domains are involved in osmosensing and/or osmoregulation of transporter activity (Tondervik and Strom, 2007; Chen and Beattie, 2008).

Sensing and signal transduction by BetP: correlation of structure with function

Biochemical data elucidated three key players for sensing and signal transduction by BetP: (i) the C-terminal domain, (ii) cytoplasmic loops, and (iii) the N-terminal domain (Fig. 8). While the N-terminal domain is not resolved in the X-ray structure of BetP (Ressl *et al.*, 2009), biochemical data concerning the functional impact of cytoplasmic loops, especially of loop 2 and loop 8, and of the

C-terminal domain on osmotic activation and transporter function can be correlated to the BetP crystal structure. Loop 2 preceding TM3 contains a cluster of negatively charged residues (Asp-131, Glu-132 and Glu-135). Mutations at Asp-131 and Glu-132 somehow prevent expression of the *betP* structural gene and a mutation at Glu-135 reduces transport activity (Ott *et al*., 2008). The BetP structure reveals an interaction of Asp-131 via a salt bridge with an Arg residue located in the C-terminal helix of the adjacent subunit, and Glu-132 with an Arg residue in loop 8, which connects TM8 and TM9 and is part of the signature sequence of the BCCT family (Kappes *et al*., 1996; Saier, 2000). Within this network, changes in orientation of the C-terminal helix in one subunit of BetP would affect the orientation and conformation of loop 2 and loop 8 in an adjacent subunit. It seems likely that such interactions would have consequences for structural features of TM3 and TM8, both of which are involved in substrate binding and Na^+ coupling. Thus, the structure of BetP suggests a 'locking' interaction of the C-terminal domain with these loops, which might translate into an inhibition of transport activity and, consequently, seems to be partly responsible for regulating the transport cycle of BetP.

Based on biochemical data, activation of BetP by a rise in the internal K^+ concentration occurs due to K^+ binding to the BetP protein at the cytoplasmic site, a fact that has so far not been confirmed by structural data. Presumably, K^+ binding influences the interaction network of the C-terminal domain with the intracellular loops 2 and 8. Furthermore, it is reasonable to assume a direct interaction of positively charged residues in the C-terminal domain with negatively charged lipids of the membrane. Lipids occupying the clefts between the individual BetP subunits or in the hydrophobic cavity in the centre of the trimer (Fig. 5) might affect the orientation of the C-terminal helices of BetP, which, in turn, might render a regulatory K^+ binding site more accessible. One can further assume that the flexible trimeric state of the transporter may be a crucial point for the activity regulation of BetP. This would involve, in all likelihood, the amphipathic helix h7 on the periplasmic side of BetP, a structure that is buried in the membrane interface (Fig. 6). A regulatory mechanism that senses differences in membrane composition or membrane state at the periplasmic side via helix h7 would be in agreement with a physical stimulus for BetP activity originating from within the membrane. Stimulus perception by helix h7 could be structurally translated into the flexible trimer by rearrangement of the BetP subunits.

The question of which transporters might form additional trimer contacts by their C-terminal domains is of interest within the context of the regulatory properties of BCCT carrier. Furthermore, it is of interest whether the C-terminal interaction with loop 2 and 8, as observed biochemically (Ott *et al*., 2008) and structurally (Ressl *et al*., 2009) in BetP, is also found in other members of the BCCT carriers. Loop 8 is nearly 100% conserved in the BCCT carriers but this is not the case for loop 2. However, six BCCT carriers (Table 1) have a negatively charged cluster at the positions of Asp-131 and Glu-132 and charged cluster in the C-terminal domain at the same position as in BetP. Two additional transporters from *C. glutamicum*, LcoP and EctP, which share with BetP the described profile of charged residues in loop2 and in their C-terminal domain, are indeed both osmoregulated transporters. Lacking glycines and prolines in their C-terminal domains, it can be assumed that these domains are α-helical and these transporters might very well be regulated in a similar way as BetP.

Subunit interactions within the trimer seem to be an essential feature of regulation of transport activity in members of the BCCT carriers. This might explain some differences in regulatory behaviour of osmoregulated BCCT carriers such as BetP compared with osmoregulated transporters of other transporter families, e.g. ProP from *E. coli* (Culham *et al*., 2008a,b) and OpuA from *L. lactis* (van der Heide and Poolman, 2000; van der Heide *et al*., 2001; Mahmood *et al*., 2009). In fact, this idea has already been suggested in a previous review (Poolman *et al*., 2004). However, the concepts derived for the osmotic activity control of transporters involved in compatible solute acquisition must be viewed with caution, since the transporter families, the structure of the OpuA, ProP and BetP transporters and their subunit composition are rather different.

Moreover, the putative primary trigger that controls transport activity in response to a high osmolarity challenge seems to be rather different as well: a change in intracellular water availability in the case of ProP (Culham *et al*., 2003), a change in the intracellular ionic strength for OpuA (van der Heide and Poolman, 2000; van der Heide *et al*., 2001; Mahmood *et al*., 2009), and altered cytoplasmic K^+ concentration in the case of BetP (Rübenhagen *et al*., 2001). Nevertheless, some common features can be deduced from the biochemical analysis the ProP, OpuA and BetP transport systems. Turgor was not found to be responsible for activation of any of these transporters; a switching behaviour of terminal domains as a mechanistic element in regulation has been suggested for all three; and an impact of membrane conditions and composition on carrier activity was found to be relevant for ProP, OpuA and BetP (van der Heide *et al*., 2001; Poolman *et al*., 2004; Schiller *et al*., 2006; Mahmood *et al*., 2009; Romantsov *et al*., 2009). It is not possible to generalize, however, on the particular nature of the transport activity-controlling stimulus, the mechanistic details of stimulus sensing, and the concept of how the transport cycle should be connected to the regulatory cycle of the various transporters under study.

The BCCT carriers: conclusions and perspectives

When initially recognized in 1996, the BCCT family of transporters comprised just four functionally characterized members (Kappes *et al*., 1996). Since then, the number of functionally studied BCCT carriers has modestly risen to 22 (Table 1). The explosion in the number of available genome sequences of microorganisms revealed a widespread occurrence of BCCT carriers (currently about 2200 representatives) that are predominantly present in members of the *Bacteria*. Substrates for BCCT carriers different from those originally found (Kappes *et al*., 1996) have also been detected in the meantime (Fig. 2). Most notably are transporters for ectoines and for DMSP.

BCCT carriers for ectoine and hydroxyectoine (Table 1), widely synthesized as osmostress and thermostress protectants by members of the *Bacteria* (Garcia-Estepa *et al*., 2006; Bursy *et al*., 2007; 2008; A.U. Kuhlmann and E. Bremer, unpubl. results), are of interest from a crystallographic point of view because the chemical structures of these compatible solutes differ considerably from those of other substrates for BCCT carriers (Fig. 2). Crystal structures of soluble ectoine and hydroxyectoine binding proteins operating in the framework of either ABC- or TRAP-type transporters have recently been determined (Hanekop *et al*., 2007; Kuhlmann *et al*., 2008; Lecher *et al*., 2009). It will be interesting to see if, similar to the situation found for the BetP transporter and the periplasmic binding protein ProX (Schiefner *et al*., 2004a; Ressl *et al*., 2009), the ligand binding site present in ectoine/hydroxyectoine-specific BCCT carriers will correspond to that of the aforementioned soluble ectoine/hydroxyectoine binding proteins.

The second type of BCCT carrier that is of considerable physiological interest is that for the compatible solute DMSP (Fig. 2), which is synthesized in huge amounts (about 1 billion tons per year) primarily by marine plankton. It can be used by microorganisms as a sulphur source and can be catabolized to the volatile dimethylsulphide, a climate-active trace gas (Dickschat *et al*., 2010; Todd *et al*., 2010; Vila-Costa *et al*., 2010). Since DMSP is also an effective microbial osmoprotectant (Pichereau *et al*., 1998), it would not be surprising if BCCT carriers for the uptake if DMSP would be discovered in addition to those involved in DMSP uptake (e.g. DddT) for catabolism (Todd *et al*., 2010).

The crystal structures of Na^+-coupled BetP transporter (Ressl *et al*., 2009) and the substrate : product antiporter CaiT (Schulze *et al*., 2010; Tang *et al*., 2010) provided the opportunity to correlate structural features of functionally distinct members of the BCCT family with a wealth of biochemical and physiological data (Peter *et al*., 1998a; Rübenhagen *et al*., 2001; Jung *et al*., 2002; Schiller *et al*., 2006; Vinothkumar *et al*., 2006; Ott *et al*., 2008; Krämer, 2009). With respect to BetP, the combination of biochemical and crystallographic analysis provided unprecedented insight into the functioning and activity control of a secondary transporter responsive to osmotic stress. The regulatory response of BetP to increased osmolarity was found to require a partly α-helical C-terminal domain of the transporter and, furthermore, evidence has been provided that the trimeric state may be a requirement for functional regulation. Based on the biochemical characteristics and crystal structures of BetP and CaiT, we suggest that many BCCT carriers should form trimeric complexes within the membrane. Our analysis of BetP and CaiT indicates that there might be a relationship between the flexibility of the BetP-type trimer and the osmotic regulation of transport activity and the rigidity of the CaiT-type trimer that is not able to regulate transport activity in response to osmotic challenges. However, we note that structural determinants of trimerization apparently show significant variation within the BCCT carriers. A combination of X-ray crystallography and biochemical analysis will be required to further unravel substrate binding, transport mechanism and activity regulation of additional members of the BCCT carriers in response to environmental stimuli.

From a crystallographic and mechanistic point of view, a high-resolution crystal structure of an H^+-coupled BetT-type choline transporter (Table 1) would be highly desirable. With the advent of such a structure, a reference protein for each of the three types of transporters grouped together into the BCCT family (Saier, 2000; 2009) would be available.

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