

The BCCT-family of carriers: from physiology to crystal structure

Journal:	<i>Molecular Microbiology</i>
Manuscript ID:	MMI-2010-09962.R1
Manuscript Type:	MicroReview
Date Submitted by the Author:	28-Jul-2010
Complete List of Authors:	Ziegler, Christine; Max Planck Institute for Biophysics, Frankfurt, Department of Structural Biology Bremer, Erhard; University of Marburg, Department of Biology Krämer, Reinhard; Universität zu Köln, Institut für Biochemie
Key Words:	osmoregulation, compatible solutes, crystal structures, ligand binding, sensing of water stress

1
2
3 **The BCCT-family of carriers: from physiology to crystal structure**

4
5 **Christine Ziegler^{1*}, Erhard Bremer^{2**} and Reinhard Krämer^{3***}**

6
7
8 ¹Max-Planck Institute for Biophysics, Max-von-Laue Str. 3, D-60438 Frankfurt, Germany

9
10 ²Philipps-University Marburg, Department of Biology, Laboratory for Microbiology, Karl-
11 von-Frisch Str. 8, D-35032 Marburg, Germany

12
13 ³University of Cologne, Institute of Biochemistry, Zùlpicher Str. 47, D-50674 Köln, Germany

14
15
16
17
18 For correspondence during the reviewing and editorial process:

19
20 Erhard Bremer: Phone (49)-6421-282 1529; Fax: (49)-6421-282 8979;

21 E-mail: bremer@staff.uni-marburg.de

22
23
24 **Key words:** Osmoregulation – compatible solutes – sodium- and proton-coupled transporters
25 - antiporters - sensing of water stress – ligand binding

26
27
28
29 _____
30 For correspondence. *E-mail christine.ziegler@biophys.mpg.de; Tel. (+49)-69-6303-3054;
31 Fax (+49)-69-6303-2209; **E-mail bremer@staff.uni-marburg.de; Tel. (49)-6421-282-1529;
32 Fax (49)-6421-282 8979; ***E-mail r.kraemer@uni-koeln.de; Tel. (+49)-221-470-6461; Fax
33 (+49)-221-470-5091

34

35 **Summary**

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

55

56

57

58

59

60

61

62

63

64

65

66

67

68

69 **Introduction**

70 One of the key parameters affecting the survival and growth of microorganisms are changes
71 in the osmotic conditions of their habitat (Kempf and Bremer, 1998; Wood *et al.*, 2001;
72 Poolman *et al.*, 2002; Altendorf *et al.*, 2009; Hagemann, 2010). Exposure of the cell to either
73 hypo- or hyper-osmotic surroundings triggers rapid water fluxes across the semi-permeable
74 cytoplasmic membrane (Fig. 1) whose direction is determined by the differential in osmotic
75 potential between the cell's interior and the environment (Wood, 1999; Bremer and Krämer,
76 2000; Altendorf *et al.*, 2009). Both the osmotically instigated water fluxes and the resulting
77 changes in turgor, an intracellular hydrostatic pressure, impinge on many physiological
78 processes (Record *et al.*, 1998; Wood, 1999). AqpZ-type aquaporins that are present in a
79 substantial number of microorganisms mediate accelerated water fluxes across the
80 cytoplasmic membrane in response to osmotic gradients (Tanghe *et al.*, 2006). However,
81 these types of water channels are not essential for water fluxes across the cell membrane in
82 bacteria. Most importantly, no single microorganism can actively transport water into or out
83 of the cell. But microorganisms can actively influence the direction of water fluxes across the
84 cell membrane in response to hyper- or hypo-osmotic conditions. They do so by indirectly
85 determining the scale of the osmotically driven water fluxes across the cytoplasmic membrane
86 by actively adjusting the osmotic potential of their interior *via* active control of their ion and
87 organic solute pools (Fig. 1).

88

89 **Environmental osmotic up- and downshifts and compensatory microbial reactions**

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

98 Exposure of a microbial cell to hyper-osmotic conditions elicits water efflux (Fig. 1),
99 thereby causing dehydration of the cytoplasm, a reduction or even collapse of turgor, and
100 eventually growth arrest (Record *et al.*, 1998). The slowing of cell growth and finally growth
101 arrest are strongly correlated with the diminished amounts of free cytoplasmic water (Cayley
102 and Record, 2003). The cell can increase the content of free cytoplasmic water by raising the

103 osmotic potential of its interior *via* accumulation of either ions or organic osmolytes (Kempf
104 and Bremer, 1998; Wood, 1999; Bremer and Krämer, 2000; Wood *et al.*, 2001; Oren, 2008,
105 Altendorf *et al.*, 2009; Hagemann, 2010).

106

107 **Properties of compatible solutes**

108 Many microorganisms accumulate a selected class of organic osmolytes, the compatible
109 solutes, to fend off the detrimental effects of high osmolarity on water content and cell
110 physiology (Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood *et al.*, 2001;
111 Empadinhas and da Costa, 2008). Compatible solutes are operationally defined as organic
112 osmolytes that can be accumulated to exceedingly high intracellular levels without impairing
113 vital functions of the cell (Kempf and Bremer, 1998; Burg and Ferraris, 2008; Empadinhas
114 and da Costa, 2008). These solutes have biophysical and biochemical properties that
115 distinguish them from other types of organic compounds, and they have been specifically
116 selected during the course of evolution as cytoprotectants in all three kingdoms of life
117 (Yancey *et al.*, 1982; Yancey, 2005). A hallmark of compatible solutes is their preferential
118 exclusion from the immediate hydration shell of proteins (Arakawa and Timasheff, 1985) due
119 to unfavourable interactions between the osmolytes and the protein backbone (Bolen and
120 Rose, 2008). This uneven distribution of compatible solutes in the cell water generates a
121 thermodynamic driving force that promotes the proper conformation of proteins and the
122 formation of protein assemblies, thereby maintaining their biological functions (Bolen and
123 Baskakov, 2001; Ignatova and Gierasch, 2006; Street *et al.*, 2006; Rosgen, 2007; Capp *et al.*,
124 2009; Rosgen, 2009; Street *et al.*, 2010). It should be noted however, that compatible solutes
125 that are ubiquitously used by microorganisms (e.g. proline and glycine betaine) interact
126 differently with protein surfaces (Auton *et al.*, 2008).

127

128 **Microbial transport systems for compatible solutes**

129 Most microorganisms that adopt the “salt-out” strategy both synthesize and acquire various
130 types of compatible solutes. Osmotically down-shocked or decaying microbial cells (e.g. after
131 viral attack), excretion products of animals and plants (urine and root exudates, respectively)
132 and of algae are sources of these compounds (Welsh, 2000). Compatible solutes are found in
133 very low concentrations (typically in the μM or nM range) in the environment. To take
134 advantage of these scarce resources as osmostress protectants, microorganisms use
135 osmotically controlled high-affinity uptake systems (Kempf and Bremer, 1998; Wood, 1999;
136 Bremer and Krämer, 2000; Wood *et al.*, 2001; Poolman *et al.*, 2004; Wood, 2006; Krämer,

137 2009; Hagemann, 2010). The intracellular level to which compatible solutes are amassed is
138 fine-tuned to the degree of osmotic stress perceived by the microbial cell. Microorganisms
139 can accumulate compatible solutes via transport systems against steep concentration
140 gradients, and intracellular concentrations above one molar can be attained (Peter *et al.*, 1996;
141 Krämer, 2009). Uptake of compatible solutes by microorganisms is energetically favourable
142 over *de novo* synthesis (Oren, 1999). Furthermore, the uptake of compatible solutes often
143 represses the expression of genes encoding enzymes required for compatible solute synthesis.
144 Transport systems for compatible solutes not only serve to capture these compounds from
145 scarce environmental sources but also serve for the re-import of these compounds leaked from
146 microbial producer cells in high osmolarity surroundings (Lamark *et al.*, 1992; Mikkat and
147 Hagemann, 2000; Grammann *et al.*, 2002).

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

164 The preferential exclusion of compatible solutes from protein surfaces is a hallmark of
165 the protective function of these types of solutes (Arakawa and Timasheff, 1985; Bolen and
166 Baskakov, 2001; Ignatova and Gierasch, 2006; Street *et al.*, 2006; Rosgen, 2007; Bolen and
167 Rose, 2008; Street *et al.*, 2010). However, in transport systems for compatible solutes, high-
168 affinity interactions between the substrates and proteins have to take place in order to archive
169 effective import. Crystallographic studies with soluble ligand-binding proteins from six ABC-
170 and two TRAP-transport systems and the secondary transporters BetP and CaiT have recently

171 provided insight into how such a task can be accomplished (Schiefner *et al.*, 2004a; Schiefner
172 *et al.*, 2004b; Horn *et al.*, 2006; Oswald *et al.*, 2008; Smits *et al.*, 2008; Ressler *et al.*, 2009;
173 Tang *et al.*, 2010; Schulze *et al.*, 2010; Wolters *et al.*, 2010). In all these systems, aromatic
174 ligand binding boxes of various architectures recognize the bulky head-groups of glycine
175 betaine, choline, L-carnitine, γ -butyrobetaine and DMSA (Fig. 2) via cation-pi interactions.
176 Hydrogen bonds and salt-bridges then fix and orient the various “tails” of the different
177 substrates (Fig. 2) within the ligand-binding site. Cation-pi interactions also play important
178 roles for the high-affinity binding of ectoine and hydroxyectoine by soluble ligand binding
179 proteins from ABC- or TRAP-transporters, but due to the chemical features of these
180 compatible solutes (Figure 2), the architecture of the ligand-binding sites is different from
181 those that are used to bind compatible solutes such as glycine betaine, proline betaine and
182 DMSA (Hanekop *et al.*, 2007; Kuhlmann *et al.*, 2008; Lecher *et al.*, 2009).

183 The types of functionally characterized microbial transporters for compatible solutes
184 include members of the multi-component ABC (e.g. ProU, OpuA, and Ehu) and TRAP
185 transporter families (Tea and Ueh) and single-component transport systems such as ProP
186 (H^+ /solute symporter; MSF-family), OpuE (Na^+ /solute symporter; SSSF-family) and
187 members of the BCCT family (e.g. BetT, BetP, OpuD). The prototypes of these osmotically
188 controlled uptake systems have been discovered through diligent genetic and physiological
189 studies with just a few bacterial species (Kempf and Bremer, 1998; Bremer and Krämer,
190 2000; Wood *et al.*, 2001; Bremer, 2002; Altendorf *et al.*, 2009; Krämer, 2009). However,
191 even a cursory inspection of the more than 1 000 microbial genome sequences available today
192 shows that compatible solute uptake systems related to those mentioned above are found
193 widely in the domain of the *Bacteria* and of the *Archaea*.

194 Three transporters have been studied in detail by biochemical, biophysical and genetic
195 approaches with the aim of unravelling the mechanism(s) and interplay of their
196 osmoregulatory and transporter function. These are the ABC transporter OpuA from
197 *Lactococcus lactis*, the H^+ /solute symporter ProP from *Escherichia coli* and the BCCT carrier
198 BetP from *Corynebacterium glutamicum* (van der Heide and Poolman, 2000; van der Heide *et al.*,
199 2001; Poolman *et al.*, 2004; Culham *et al.*, 2008a; Culham *et al.*, 2008b; Krämer, 2009;
200 Mahmood *et al.*, 2009).

201 The BCCT carrier BetP from *C. glutamicum* occupies a special position within this
202 group of osmoregulatory compatible solute transport systems because a well-resolved crystal
203 structure of the BetP protein with bound glycine betaine and sodium ligands has been
204 reported (Ressler *et al.*, 2009). Most recently, three X-ray structures of another BCCT-carrier,

205 the L-carnitine/ γ -butyrobetaine antiporter CaiT from *E. coli* and *Proteus mirabilis* was solved
206 in complex with its substrate L-carnitine and γ -butyrobetaine (Tang *et al.*, 2010; Schulze *et*
207 *al.*, 2010). The CaiT proteins from these two organisms are not involved in osmotic stress
208 responses and instead mediate L-carnitine: γ -butyrobetaine antiport under anaerobic growth
209 conditions (Jung *et al.*, 2002).

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

220

221 **The BCCT carriers: discovery and general features**

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

234 Using the substrates of the BetT, CaiT, OpuD and BetP transporters as a point of
235 reference, Milton Saier's laboratory subsequently grouped these transporters into the BCCT
236 family (betaine-choline-carnitine-transporter) (Saier, 2000) and placed them into sub-group
237 2.A.15 of the International Union of Biochemistry and Molecular Biology-approved
238 transporter classification (TC) system [<http://www.tcdb.org>] (Saier *et al.*, 2009). BCCT

239 carriers comprise uptake systems that are energized either by proton-motive-force-driven (e.g.
240 BetT) or sodium-motive-force-driven (e.g. BetP) symport, or more rarely, by
241 substrate:product antiport (e.g. CaiT) (Table 1).

242 It was originally thought that a unifying feature of BCCT carriers is the transport of
243 substrates with quaternary ammonium groups $[R-N^+(CH)_3]_3$ such as choline, L-carnitine and
244 glycine betaine (Kappes *et al.*, 1996; Saier, 2000) (Fig. 2). In recent years, several new
245 substrates for BCCT carriers have been discovered. Of these, proline betaine, acetylcholine,
246 dimethylsulfoniopropionate (DMSP) and dimethylsulfonioacetate (DMSA) all possess fully
247 methylated nitrogen- or sulfonium headgroups (Fig. 2) and, thus, conform in a wider sense to
248 the originally suggested chemical features of substrates for BCCT carriers. However, the
249 chemical structures of the compatible solutes ectoine and hydroxyectoine (Fig. 2), substrates
250 for BCCT carriers detected in various microbial species (Table 1), do not match this general
251 scheme. In addition, some BCCT-type transporters also mediate proline uptake, but usually
252 with rather low affinity. So far, no functionally characterized BCCT carrier has been
253 described to catalyze the uptake of the full range of currently known substrates (Table 1; Fig.
254 2). However, for some BCCT carriers competition experiments have been carried out that
255 suggests a rather restricted substrate specificity for a given BCCT carrier. For instance, in the
256 halotolerant alkaliphilic cyanobacterium *Aphanothece halophytica*, the BetT-mediated
257 glycine betaine transport (Table 1) was not inhibited by glycine betaine aldehyde,
258 dimethylglycine, sarchosine, choline or proline (Laloknam *et al.*, 2006).

259

260 **Physiological functions of BCCT carriers and transcriptional regulation of their** 261 **structural genes**

262 The explosion in the number of available genome sequences of *Bacteria* and *Archaea*
263 revealed a widespread distribution of BCCT carriers in the microbial world. Under the entry
264 “IPR000060 BCCT transporter”, the InterPro database [<http://www.ebi.ac.uk/interpro/>] lists
265 currently about 2 200 protein sequences. Most of these protein sequences originate from
266 members of the *Bacteria* and only 22 are from members of the *Archaea*. The InterPro
267 database also records a limited number of BCCT carriers from *Eukarya*, but nothing is known
268 about their function.

269 Most BCCT carriers have a common hydropathy profile with 12 predicted trans-
270 membrane (TM)-spanning segments and exhibit N- and C-terminal extensions of various
271 lengths (Table 1) that are predicted to protrude into the cytoplasm. An important functional
272 role of these N- and C-terminal domains for the control of transport activity and osmotic

273 regulation was first discovered for the glycine betaine transporter BetP (Peter *et al.*, 1998a).
274 Subsequent studies with the choline transporters BetT of *E. coli* (Tondervik and Strom, 2007)
275 and *Pseudomonas syringae* (Chen and Beattie, 2008), revealed the contribution of the C-
276 terminal domain for the proper functioning of these BCCT carriers as well. The most
277 conserved part of BCCT carriers comprises TM8 and the connecting loop to TM9 and these
278 segments contain the tryptophane motif that is the signature sequence of BCCT carriers
279 (Kappes *et al.*, 1996; Saier, 2000).

280 In the Pfam database [<http://pfam.sanger.ac.uk/>] some physiologically uncharacterized
281 BCCT carrier related proteins are listed that possess additional domains (Usp, CoaE,
282 TrkA_C) or membrane-spanning segments fused to the canonical 12 TM core structure of
283 BCCT carriers. Twenty-two microbial BCCT carriers have so far been functionally
284 characterized at least to some extent (Table 1). Using the *C. glutamicum* BetP glycine betaine
285 transporter protein as the search template, these 22 proteins exhibit a range from 48% down to
286 25% identical amino acid residues (Table 1). However, it should be noted that functionally
287 uncharacterized BCCT carriers can readily be found in database searches that are much more
288 closely related to the BetP protein from *C. glutamicum* than those listed in Table 1.

289 Only two of the functionally characterized transporters of the BCCT-family are
290 physiologically not connected with microbial osmotic adjustment processes: the CaiT
291 proteins from *E. coli* and *Proteus mirabilis* (Table 1). The CaiT proteins are unusual members
292 of the BCCT-family, since they do not require a sodium- or proton-gradient to fuel transport
293 and instead function according to the substrate:product antiport principle. L-carnitine (Fig. 2)
294 is taken up in *E. coli* via CaiT under anaerobic growth conditions when no other electron
295 acceptor or glucose is present and it is then metabolized to γ -butyrobetaine (Fig. 2) that is
296 subsequently excreted via the CaiT antiporter (Jung *et al.*, 2002). The genetic regulation of
297 the *caiTABCDE* gene cluster reflects this physiological role. It is controlled by the globally
298 acting regulators CRP and FNR (both positively acting) and the nucleoid-associated protein
299 H-NS (negatively acting) of *E. coli* and by the carnitine-responsive activator protein CaiF
300 (Buchet *et al.*, 1998). A BCCT carrier (DddT) was recently shown to mediate uptake of
301 sulfur-containing compatible solute dimethylsulfoniopropionate (DMSP) (Fig. 2) in a relative
302 of *Halomonas venusta* (Todd *et al.*, 2010). DMSP is widespread in marine environments and
303 can be used by microorganisms as an effective osmoprotectant or as a versatile sulfur source
304 (Pichereaux *et al.*, 1998; Dickschat *et al.*, 20110; Vila-Costa *et al.*, 2010). DddT-mediated
305 uptake of DMSP in the studies *Halomonas strain* is connected with catabolism since

306 expression of the *dddT* gene is induced by DMSP and two of its metabolites (Todd *et al.*,
307 2010).

308 The majority (19 out of 22) of the currently functionally characterized BCCT carriers
309 (Table 1) are involved in microbial adjustment to high osmolarity surroundings *via* uptake of
310 compatible solutes or their biosynthetic precursors. In line with these physiological functions,
311 expression of their structural genes is typically induced in cells grown at high osmolarity.
312 With the exception of the proton-coupled choline transporter BetT from *E. coli* and from
313 *Pseudomonas syringae* and CutT from *Staphylococcus xylosus* (Lamark *et al.*, 1991;
314 Rosenstein *et al.*, 1999; Tondervik and Strom, 2007; Chen and Beattie, 2008), substrate
315 uptake via BCCT carriers is driven by sodium symport (Table 1). As an example, BetP-
316 mediated import of one molecule of glycine betaine is accompanied by the uptake of two
317 sodium ions (Farwick *et al.*, 1995; Peter *et al.*, 1996). Since glycine betaine concentration
318 above 1 mol/l can be attained by *C. glutamicum*, massive amounts of Na⁺ ions will be pumped
319 into the cell under osmotic stress conditions. It is apparent that the functioning of Na⁺-driven
320 BCCT carriers for osmoprotectants (Table 1) must be physiologically coupled to the
321 functioning of Na⁺ extrusion systems in order to keep the intracellular concentration of this
322 cytotoxic cation low (Padan *et al.*, 2005).

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

332 It is generally not firmly understood at the molecular level how bacteria sense osmotic
333 stress and how this environmental cue ultimately results in altered gene transcription (Bremer
334 and Krämer, 2000; Cheung *et al.*, 2003; Nagarajavel *et al.*, 2007; Romeo *et al.*, 2007;
335 Rosenthal *et al.*, 2008; Altendorf *et al.*, 2009; Krämer, 2010). Information on osmotically
336 controlled gene expression is available for only a few of the functionally characterized BCCT
337 carriers (Table 1). For instance, osmotic induction of *betP* transcription is governed by the
338 two-component MtrAB regulatory system of *C. glutamicum* (Moker *et al.*, 2004; Moker *et al.*,
339 2007). Expression of the *B. subtilis opuD* gene is partially dependent on the alternative

340 transcription factor SigB (F. Spiegelhalter and E. Bremer; unpublished data), the master
341 regulator of the general stress regulon. SigB also controls the osmotic induction of *ectT* from
342 *Virgibacillus pantothenicus* (Kuhlmann *et al.*; unpublished data). The substrates of
343 osmotically induced BCCT carriers typically do not trigger gene expression of their
344 corresponding structural genes, with the exception of the choline transporters BetT from *E.*
345 *coli* (Lamark *et al.*, 1996) and CudT from *Staphylococcus xylosus* (Rosenstein *et al.*, 1999)
346 where unrelated choline-responsive repressor proteins (BetI and CudC) have been identified.
347 Osmoregulation and simultaneous substrate induction of *betT* and *cudT* transcription is of
348 physiological relevance because the function of choline as an osmoprotectant is entirely
349 dependent on its conversion into glycine betaine.

350 It is increasingly recognized that compatible solutes not only function as microbial
351 osmoprotectants, but serve other stress protective functions as well (Yancey, 2005;
352 Empadinhas and da Costa, 2008). In particular, a compatible-solute-mediated temperature
353 stress protection both at the upper and lower boundaries of growth has been observed in
354 various microbial species. BCCT carriers participate in these processes in several
355 microorganisms. Examples are the BetT-mediated uptake of choline in *E. coli* (Caldas *et al.*,
356 1999) and the OpuD-mediated uptake of glycine betaine in *B. subtilis* as heat stress
357 protectants (Holtmann and Bremer, 2004), the chill stress protection by glycine betaine *via*
358 BetL-mediated import in *Listeria monocytogenes* (Angelidis and Smith, 2003; Sheehan *et al.*,
359 2006) and OpuD in *B. subtilis* (Hoffmann and Bremer; unpublished data) and the EctT-
360 mediated uptake of ectoine/hydroxyectoine in *V. pantothenicus* (Kuhlmann *et al.*;
361 unpublished data).

362

363 **The BetP transporter from *C. glutamicum* and other BCCT carriers: structure, function** 364 **and regulation**

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

366 In comparison to other bacteria such as *E. coli* (Altendorf *et al.*, 2009) or *B. subtilis* (Bremer,
367 2002), which employ a combination of primary transporters (the ABC transporters) and
368 secondary transporters, uptake of compatible solutes in *C. glutamicum* is mediated entirely by
369 osmoregulated secondary transporters (Peter *et al.*, 1998b). Three of them, BetP, EctP, and
370 LcoP, are members of the BCCT family. These transporters vary in substrate specificity,
371 affinity, and transcriptional regulation, but all respond to hyperosmotic stress by regulating
372 the level of transport activity (Krämer, 2009). BetP is by far the best described, both with
373 respect to transport and regulation. BetP is specific for glycine betaine and couples its uptake

374 to the electrochemical Na^+ potential (smf) by co-transport with two Na^+ ions, leading to
375 extremely high accumulation ratios up to 10^6 and internal concentrations above 1 mol/l
376 (Farwick *et al.*, 1995; Peter *et al.*, 1996).

377 The activity of BetP is close to zero in the absence of hyperosmotic stress. Upon
378 osmotic challenge, BetP starts taking up betaine in less than a second (Peter *et al.*, 1998a).
379 When transport activity is plotted against the extent of osmotic stress, a steep sigmoidal
380 increase in activity is observed until an optimum is reached; in intact *C. glutamicum* cells this
381 occurs around 1.3 osmol/kg (Fig. 3). Consequently, besides its catalytic activity (Na^+ -coupled
382 glycine betaine uptake), BetP acts both as an osmosensor and an osmoregulator by responding
383 to osmotic stress with instant regulation of its transport activity. This fact was documented
384 both by heterologous expression of the *betP* gene in *E. coli* (Peter *et al.*, 1996), and by
385 reconstitution of the purified BetP protein in proteoliposomes (Rübenhagen *et al.*, 2000). In
386 these two artificial membrane surroundings, the BetP transporter from *C. glutamicum* retained
387 all its functions, indicating that no other proteinaceous activating factors are involved in
388 controlling BetP activity. The differences in the activation pattern, observed in Fig. 3, can be
389 explained by a significantly different share of negatively charged phospholipids in the
390 membranes of the two different organisms (Schiller *et al.*, 2006).

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

406

407

408 **X-ray structures of BetP and CaiT: structural models for members of the BCCT family**
409 BCCT carriers involved in osmotic stress response catalyze Na⁺- or H⁺-symport, during
410 which the smf or pmf drives the conformational change from an outward-facing to an inward-
411 facing state according to the alternating access mechanism of secondary transporters
412 (Jardetzky, 1966) (Fig. 4). Structural data are required in order to understand the mechanisms
413 of ion-coupled osmolyte symport by BCCT carriers. Crystal structures of two members of this
414 transporter family are available to date: the structure of BetP from *C. glutamicum*, which was
415 solved by X-ray crystallography to 3.35 Å resolution in complex with its substrate glycine
416 betaine (Ressl *et al.*, 2009) [PDB entry 2WIT], a 3.15Å structure of CaiT from *E. coli* in
417 complex with four L-carnitine molecules (Tang *et al.*, 2010) [PDB entry 3HFX], a 3.5 Å
418 structure of CaiT from *E. coli* in complex with two γ -butyrobetaine molecules [PDB code:
419 2WSX], and an apo structure of CaiT [PDB entry 2WSW] from *P. mirabilis* at 2.3Å (Schulze
420 *et al.*, 2010). Although CaiT shares the least sequence identity to BetP of all functionally
421 characterized BCCT carriers listed in Table 1, the crystal structures of BetP and CaiT
422 superimpose quite well with an r.m.s.d. deviation of ~2.5 Å. Both the purified BetP and CaiT
423 proteins form trimers (Jung *et al.*, 2002; Ziegler *et al.*, 2004; Vinothkumar *et al.*, 2006) and
424 both proteins also crystallize as trimers (Fig. 5). Each subunit consisting of twelve TM α -
425 helices and contain a curved amphipathic α -helix (helix 7) that runs parallel to the plane of the
426 cytoplasmic membrane and a long bend TM helix (TM2), which both provide a hydrophobic
427 scaffold surrounding the centre of the transporter (Fig. 6). Helix 7 is located at the trimer
428 centre of both the BetP and CaiT transporters, close to the periplasmic membrane surface,
429 where it mediates contacts between all three subunits within the trimer at the periplasmic side.

430 A feature that distinguishes BetP from CaiT is a α -helical C-terminal domain
431 protruding into the cytoplasm (Fig. 5B). In BetP, the C-terminal domain on the cytoplasmic
432 side contributes to trimer contacts and as a result, a slightly different subunit arrangement
433 within the trimers of the BetP and CaiT crystal structures are observed (Fig. 5B). In
434 comparison to BetP, each subunit within the trimer assembly is tilted somewhat resulting in a
435 larger cavity in the trimer centre of CaiT (Fig. 5B, black arrow). TM3, TM4, TM8 and TM9
436 form an iris-shaped 4-helix bundle close to the centre of both the BetP and CaiT subunit, with
437 aromatic side chains in TM4 and TM8 contributing to the substrate-binding site (Fig. 7B).

438 The 3.15 Å crystal structure of CaiT from *E. coli* reveals four L-carnitine molecules
439 (Fig. 7A) most likely because the protein was crystallized with an excess of L-carnitine. The
440 primary binding site at the center of the protein and the secondary substrate-binding site at the
441 bottom of the intracellular vestibule have been confirmed by site-directed mutagenesis studies

442 (Tang *et al.*, 2010), while the two others L-carnitine molecules might be associated with the
443 crystallized CaiT protein for structural reasons. The ligand-binding site in one of the *E. coli*
444 CaiT structure contains a bound γ -butyrobetaine (Schulze *et al.*, 20210), the substrate that is
445 exported from the *E. coli* cell after metabolism of L-carnitine under anaerobic growth
446 conditions (Eichler *et al.*, 1994; Jung *et al.*, 2002). As observed in the CaiT structure in
447 complex with L-carnitine, the bulky headgroup of γ -butyrobetaine is coordinated by cation- π
448 interactions and van-der-Waals contacts. In contrast, the central binding site of the *P.*
449 *mirabilis* CaiT structure does not contain any bound substrate. A second γ -butyrobetaine
450 molecule is bound in the extracellular cavity of the *E. coli* CaiT protein (Fig. 7A). Instead of
451 γ -butyrobetaine, two ordered water molecules occupy the corresponding position in the *P.*
452 *mirabilis* CaiT protein, most likely because access of the substrate is blocked by crystal
453 contacts. The functional role of this putative second ligand-binding site is unclear. The
454 different CaiT crystal structures revealed that two of the L-carnitine binding sites are identical
455 to those containing γ -butyrobetaine, while the other two L-carnitine sites most likely contain
456 non-specifically bound substrate (Tang *et al.*, 2010; Schulze *et al.*, 2010).

457 The central binding site (Fig. 7B) of L-carnitine and γ -butyrobetaine in CaiT
458 correspond to the glycine betaine-binding site present in BetP (Ressl *et al.*, 2009). However,
459 the bound L-carnitine and γ -butyrobetaine molecules in the CaiT structures are accessible
460 from the cytoplasm, while in the BetP structure, the access to the bound glycine betaine
461 molecule is occluded from both sides of the membrane (Fig. 7C). In TM4 and TM8 of BCCT
462 carriers, highly conserved aromatic residues, including those that are part of the BCCT
463 signature sequence (Kappes *et al.*, 1996; Saier, 2000), stabilize the 4 TM helix bundle in the
464 CaiT and BetP subunits. The amino acid sequence of TM3 in the BetP and CaiT proteins is
465 not conserved; however, both crystal structures revealed that this trans-membrane region has
466 a locally unwound segment approximately halfway across the membrane (Fig. 7A). In BetP,
467 TM3 harbours one of the Na⁺ ion binding sites. This sodium ion contributes to the
468 coordination of the carboxyl group of glycine betaine (Fig. 2) and, thereby, stabilizes the
469 ligand within its binding site (Ressl *et al.*, 2009), while the second sodium ion stabilizes the
470 unwound segment of TM3. Unlike BetP, CaiT is a Na⁺-independent transporter and the
471 functional role of the locally unwound segment in TM3 is therefore not immediately obvious.
472 However, two residues present in TM3 of CaiT are involved in interactions with the L-
473 carnitine molecule trapped in both the primary and secondary binding site. Interestingly, an
474 Arg residue in TM7 in CaiT occupies the position of the second Na⁺-binding site present in
475 BetP. Similar to Na⁺, the positive charge of the Arg amino acid side chain stabilizes the

476 unwound helix in TM3. The coordination of the carboxyl group of the γ -butyrobetaine ligand
477 is maintained in CaiT by the sulfur of a Met residue that is highly conserved in CaiT-related
478 proteins but it is absent in BetP-related proteins.

479 The crystal structures of BetP (Ressl *et al.*, 2009) and of CaiT (Tang *et al.*, 20210:
480 Schulze *et al.*, 2010) share a strikingly similar architecture with that of other Na⁺-coupled
481 symporters like LeuT (Yamashita *et al.*, 2005; Singh *et al.*, 2007; Zhou, 2007; Singh *et al.*,
482 2008), vSglT (Faham, 2008) and Mhp1 (Weyand, 2008), with H⁺-coupled symporters like
483 ApcT (Shaffer *et al.*, 2009) and antiporters like AdiC (Gao *et al.*, 2010), despite the fact that
484 they are not related at the amino acid sequence level. Each of these transporters possesses a
485 core domain consisting of 10 TM helices. Hydrophathy profile alignments (Lolkema and
486 Slotboom, 2008) confirm the structural similarity between transporters of the
487 neurotransmitter:sodium:symporter family (LeuT), the sodium:solute:symporter family
488 (vSglT), and the aminoacid:polyamine:organocation family (ApcT) (Gouaux, 2009;
489 Krishnamurthy *et al.*, 2009; Shaffer *et al.*, 2009). These ten C-terminal TMs (corresponding
490 to TM3-12 in BetP) contain an inverted repeat of two 5 TM helices each. Repeat 1 (TM3-
491 TM7 in BetP) is a structural homolog of repeat 2 and they are related by pseudo two-fold
492 symmetry axis running parallel to the membrane (Fig. 6B). Substrate and co-substrate binding
493 sites are all located in the interface between the two intertwined repeats. A superposition of
494 the core structures of this group of transporters reveals an r.m.s.d. of 3.8–4.5 Å, indicating not
495 only a high degree of structural conservation with respect to the overall fold but also to the
496 position of the substrate and co-substrate binding sites (Caplan *et al.*, 2008; Noskov and
497 Roux, 2008; Ressl *et al.*, 2009). The common core structure, the similar location of the
498 substrates in these transporters, and the fact that the crystal structures reflect distinct
499 conformations, were recently used to propose a common molecular mechanism for Na⁺- and
500 H⁺-coupled symport and antiport (Abramson and Wright, 2009; Gouaux, 2009;
501 Krishnamurthy *et al.*, 2009; Shaffer *et al.*, 2009).

502 The Na⁺-coupled glycine betaine symporter BetP and the L-carnitine/ γ -butyrobetaine
503 antiporter CaiT represent different subgroups within the BCCT carriers (Table 1). However,
504 the striking similarities of their crystal structures provide now a solid basis for formulating
505 unifying concepts that describe functional mechanisms operating within the BCCT family. In
506 our comparisons of a large number of BCCT carriers, we noted a high amino acid sequence
507 conservation of those residues that are involved in coupling and substrate binding within the
508 BCCT family. The degree of sequence identity in TM3, TM4 and TM8 is very high within the
509 BCCT family. However, there are two distinct pattern of conservation: (a) residues involved

510 in ion-coupling or counter-substrate coupling and (b) residues involved in binding of the fully
511 methylated head group of various compatible solutes (Fig. 2 and 7B). Therefore, the
512 structures of BetP and CaiT probably can be used to explain transport of other methylated
513 ammonium or sulfur compounds such as choline and DMSP and permits structure-guided
514 speculations on the transport mechanism of non-quaternary ammonium substrates of BCCT
515 carriers such as ectoine and hydroxyectoine (Fig. 2). Thus, the BetP and CaiT structures allow
516 us to search for key residues that are likely to be involved in functionally relevant
517 conformational changes in BCCT carriers.

518

519 **Substrate binding: what determines substrate specificity and coupling mode in the** 520 **BCCT family?**

521 In general, it is not possible to predict with confidence the substrate profile of a given BCCT
522 carrier solely by inspecting and aligning amino acid sequences. However, the BetP and CaiT
523 structures reveal a striking pattern in substrate binding that allows one, at least to some extent,
524 to predict the substrate profile of other BCCT carriers based on sequence alignments. Glycine
525 betaine transporters of the BCCT family show a substantial degree of sequence relatedness
526 (between 48-40%) in the 10 TM transporter core (excluding the N-terminal and C-terminal
527 extensions). In contrast, the amino acid sequence relatedness of the 10 TM core falls below
528 40% for ectoine and choline transporters and even lower (below 30 %) for L-carnitine/ γ -
529 butyrobetaine antiporters.

530 The BetP crystal structure revealed the architecture of the binding site for its ligand
531 glycine betaine and the functional importance of key-residues within this binding site were
532 confirmed by site-directed mutagenesis studies (Ressl *et al.*, 2009). The architecture of the
533 ligand-binding site present in BetP is nearly identical with the primary ligand-binding site for
534 L-carnitine and γ -butyrobetaine in CaiT (Tang *et al.*, 20210; Schulze *et al.*, 2010). Hence, the
535 crystal structures of BetP and CaiT revealed for the first time the molecular determinants for
536 substrate binding of trimethylammonium compounds by members of the BCCT carriers. In
537 particular, the indole groups of three tryptophane residues, one positioned in TM4 and two
538 positioned in TM8, are arranged in form of an aromatic cage in which the fully methylated
539 head groups of glycine betaine, L-carnitine and γ -butyrobetaine (Fig. 2) are stabilized *via*
540 cation-pi interactions. The carboxyl groups of these ligands protrude out from this aromatic
541 cage and are coordinated *via* hydrogen bonding interactions within the ligand-binding site
542 (Fig. 7B). The coordination of the glycine betaine, L-carnitine and γ -butyrobetaine substrates
543 within the BetP and CaiT transporters show striking similarities to that found in the ligand-

544 binding proteins (ProX) of the glycine betaine ABC-type uptake system from *E. coli*
545 (Schiefner *et al.*, 2004a) and the hyperthermophilic archaeon *Archeoglobus fulgidus*
546 (Schiefner *et al.*, 2004b). The ProX proteins are soluble and function in the context of ABC-
547 transporters and are thus not evolutionarily closely related to the integral membrane proteins
548 BetP and CaiT. Hence, nature has used common design principles to achieve the selective and
549 high-affinity binding of this group of compatible solutes by proteins, compounds which are
550 typically preferentially excluded from the hydration shell of the protein backbone (Arakawa
551 and Timasheff, 1985; Bolen and Baskakov, 2001; Street *et al.*, 2006; Rosgen, 2007; Bolen
552 and Rose, 2008; Street *et al.*, 2010). The crystal structures of ligand-binding proteins from
553 ABC- and TRAP-transport systems revealed that the binding of compatible solutes in
554 substrate-selective binding sites present in transporter proteins seems to require a certain
555 number of aromatic residues for substrate coordination *via* cation- π and van der Waals
556 interactions in order to provide a surface that minimally repels osmolytes (Schiefner *et al.*,
557 2004a; Horn *et al.*, 2006; Hanekop *et al.*, 2007; Kuhlmann *et al.*, 2008; Smits *et al.*, 2008;
558 Capp *et al.*, 2009; Lercher *et al.*, 2009; Ressler *et al.*, 2009; Tang *et al.*, 20210; Schulze *et al.*,
559 20210; Wolters *et al.*, 2010). Several crystal structures of binding proteins from ABC- or
560 TRAP-type transport systems in complex with compatible solutes used by BCCT carriers
561 (Table 1) have been reported (Schiefner *et al.*, 2004a; Schiefner *et al.*, 2004b; Horn *et al.*,
562 2006; Hanekop *et al.*, 2007; Kuhlmann *et al.*, 2008; Oswald *et al.*, 2008; Smits *et al.*, 2008;
563 Lecher *et al.*, 2009; Wolters *et al.*, 2010). Hence, the coordination of the substrates in these
564 soluble proteins might give valuable clues to substrate binding site(s) present in BCCT
565 carriers for which no crystal structure is yet available (e.g. transporters for ectoines and
566 DMSP).

567 Most of the BCCT carriers show a high degree of conservation of the Trp residues in
568 TM4 and TM8 that coordinate the trimethylammonium ligands in BetP and CaiT (Ressler *et al.*,
569 2009; Tang *et al.*, 2010; Schulze *et al.* 20210) but these residues can not be identified in
570 ectoine/hydroxyectoine transporters of the BCCT-family. From these patterns of
571 conservation, we conclude that the coordination of ectoine or hydroxyectoine, which lack a
572 quaternary ammonium group (Fig. 2), is different from that of glycine betaine, γ -
573 butyrobetaine or L-carnitine, while coordination of choline is likely to be similar. This latter
574 suggestion is supported by the molecular determinants governing choline binding by the
575 periplasmic ligand binding ChoX protein from *Sinorhizobium meliloti* that also archives the
576 binding of the methylated head group of choline through cation- π interactions in an aromatic
577 ligand-binding cage (Oswald *et al.*, 2008).

578 Like glycine betaine, ectoine and hydroxyectoine carry a negatively charged
579 carboxylate (Fig. 2). Hence, it is tempting to speculate that a sodium ion might interact
580 directly with the carboxylate of ectoine and hydroxyectoine in a similar way to that found in
581 BetP (Ressl *et al.*, 2009). The coordinating Na⁺ ion in the BetP crystal structure is located
582 halfway across TM3, close to an unwound, glycine-rich segment that separates TM3 into two
583 segments (Fig. 7B). This flexible segment in BetP contains a GMGIG motif that is conserved
584 in glycine betaine-specific transporters of the BCCT family. TM3 in the Na⁺-independent
585 substrate:product antiporter CaiT shows no sequence homology in this region, while
586 ectoine-specific BCCT carriers show sequence modifications at the corresponding position.
587 Substrate-coordination by residues in TM3 might also be crucial for proton-coupled BetT-like
588 proteins of the BCCT family. The chemical nature of glycine betaine and choline differs with
589 respect to their carboxyl and alcohol groups (Fig. 2). While salt bridges to the substrate are
590 maintained in Na⁺-coupled transporters by the coupling ion, this might not be the case for
591 choline, which carries a positive charge (Fig. 2). However, the choline transporter BetT from
592 *E. coli* comprises a charged residue in the flexible segment in TM3. The presence of a side
593 chain capable of reversible protonation during the transport cycle might be a pre-requisite for
594 the H⁺-coupled transport, since such an arrangement was also observed in the H⁺-coupled
595 amino acid transporter ApcT from *Methanocaldococcus jannaschii* (Shaffer *et al.*, 2009).

596 There might be substrates for BCCT carriers that are different from those compiled in
597 Fig. 2. Some putative BCCT carriers harbour conserved, but significant different motifs of the
598 glycine betaine, choline, ectoine or carnitine transporters of the BCCT-family. This
599 functionally uncharacterized group of BCCT carriers is predominantly found in members of
600 the *Firmicutes*, and they also form the major group of BCCT carriers present in
601 *Synechococcus*, a subclass of cyanobacteria. Depending on the degree of their salt stress
602 resistance these microorganisms synthesize sucrose, trehalose, glucosylglycerol and glycine
603 betaine under salt stress (Hagemann, 2010). Some marine *γ-Proteobacteria* possess BCCT
604 carriers with a very low degree of sequence homology to BetP (approximately 20%) and,
605 most strikingly, they lack TM1. Accordingly, these transporters have a predicted
606 transmembrane topology that is different from that of BetP. These considerations suggests
607 that it might be rewarding to study these so-far uncharacterized BCCT carriers with respect to
608 their potential substrates, their transport activity profiles and topological organization within
609 the cytoplasmic membrane

610

611

612 **The oligomeric state of BCCT carriers and their terminal domains**

613 BetP and CaiT are both trimeric in detergent solution (Ziegler *et al.*, 2004; Vinothkumar *et al.*, 2006), as well as in the membrane when analyzed by 2D electron crystallography, and in
614 the 3D crystal (Ressl *et al.*, 2009; Tang *et al.* 2010; Schulze *et al.*, 2010; Tsai *et al.*,
615 submitted). The observed trimer formation is a consequence of the unique structural elements
616 characterizing the fold of the BCCT carriers, which are the amphiphatic helix 7 and the long
617 bend TM2. They provide a hydrophobic interface, which force a triangular subunit
618 arrangement for stability reasons. In fact, BetP and CaiT have proven to be very stable both in
619 detergent solution and when reconstituted back into the membrane (Tsai *et al.* 2007). It is thus
620 tempting to speculate that trimer-formation is a general feature of the BCCT carriers with
621 functional impact for transport and/or regulation. We have analyzed the differences in trimer
622 contacts in the osmotically regulated BetP and the osmotically non-regulated CaiT proteins to
623 validate this assumption. The three subunits within the CaiT and BetP trimer form a central
624 conical cavity lined by aromatic side chains from TM2, TM3 and h7 (Fig. 5A). The
625 hydrophobic cavity in the trimer centre is most likely filled with lipid as observed for the
626 crystallized BetP protein (Ressl *et al.*, 2009). Residues contributing to the formation of this
627 cavity are highly conserved in members of the BCCT carriers. On the periplasmic side, the
628 amphipathic helix 7 is the key player in maintaining trimer contacts (Fig. 6). Helix h7 in BetP
629 and CaiT is rich in Leu and Asp residues and shows characteristics of a non-specific lipid
630 interaction site that stabilizes the trimer assembly. This helix represents a structurally distinct
631 feature of the BCCT carriers since it is not present in any other transporter sharing the same
632 overall inverted structural repeat (e.g. LeuT). In BetP and CaiT, helix h7 separates the two
633 structural repeats from each other and, therefore, cannot be attributed to either of the repeat
634 parts of the transporter proteins. Contacts involve mainly hydrophobic residues in helix h7,
635 TM2, TM3 and loop7 of adjacent subunits. Residues participating in trimer contact via helix
636 h7 are not entirely conserved within the BCCT carriers. CaiT shares only 17% sequence
637 identity in this region to the BetP protein. However, the two structurally important polar
638 interactions observed in BetP are also found in CaiT and are predicted to occur in eight other
639 BCCT carriers listed in Table 1. In CaiT and nine other BCCT carriers, additional subunit-
640 subunit contacts are likely to be promoted by ionic interactions, resulting in a quite rigid
641 trimer assembly; in contrast, in BetP they are formed by more flexible hydrophobic
642 interactions. However, only BetP_{Cg} and OpuD_{Vc} (Table 1) seem to have exclusively
643 hydrophobic interactions in their trimer contact regions as found in BetP. The majority of
644 BCCT carriers show (i) a conserved length of h 7 and (ii) the potential for both hydrophobic
645

646 and ionic interactions *via* helix h7. Both parameters favour a triangular interface, while a
647 dimer or a tetramer formation is not possible. Therefore we can safely assume that the
648 majority of BCCT carriers form trimers in the plane of the cytoplasmic membrane. It seems
649 that BetP and CaiT represent two different structural solutions within the BCCT carriers to
650 foster trimerization.

651 It is an intriguing question whether the flexible trimer interactions observed in BetP in
652 comparison to those found in CaiT can be related to BetPs osmoregulatory abilities. Subunit
653 interactions within the BetP trimer not only involve helix h7 but also involve the C-terminal
654 helix of each BetP subunit within the trimer assembly; this C-terminal extension interacts
655 with loop 2 and the C-termini of the other two BetP subunits. Only when the subunits are
656 arranged in a trimer, individual C-terminal domains of BetP would not hinder each other
657 sterically. The C-terminal helix of BetP is not found in CaiT (Fig. 5). The additional trimer
658 contact formed by the C-terminal helix of BetP within the BetP trimeric structure is of
659 particular interest because biochemical studies have provided compelling evidence for the
660 involvement of this domain in osmosensing and regulation (Ott *et al.*, 2008). The C-terminal
661 domain of BetP contains several positively charged residues clustered at the N-terminal part
662 of the C-terminal domain (cluster 1: R554, R558, R565, R567, and R568) and near the end of
663 the C-terminal domain (cluster 2: R576, K581, R582-584, R586, and K587). It lacks Gly
664 and/or Pro residues, except close to its very end, and thus forms a straight α -helix (Fig. 5B
665 and 6B). Biochemical data revealed the functional importance of the conformation and
666 position of the first charge cluster within the C-terminal domain of BetP (Ott *et al.*, 2008;
667 Ressler *et al.*, 2009). In the context of trimer contacts in BetP, arginines in the first cluster form
668 salt bridges to a glutamate separating both clusters in the C-terminal domain of the adjacent
669 subunit. In the 22 functionally characterized microbial BCCT carriers (Table 1), the C-
670 terminal domains do not have a conserved sequence, but three different motifs can be
671 observed: (i) BetP-type C-terminal domains feature two clusters with opposite charges
672 positioned several residues apart from each other, but lack flexible stretches of Gly and/or Pro
673 residues. (ii) CaiT-type C-terminal domains have, at most, very short charged clusters.
674 Consequently, the BetP-type C-terminal domains present in BetP_{Cg}, BetT_{Ah}, CudT, BetH_{Ht},
675 OpuD_{Bs}, EctM, ButA and EctP (Table 1) are likely to contribute to trimer contacts, while
676 CaiT-type C-terminal domains present in BetM and DddT (Table 1), do not do so. (iii) There
677 is a third type of C-terminal domain in members of the BCCT carriers. It consists of charged
678 clusters but is also rich in Gly and/or Pro residues, suggesting that these C-terminal domains
679 are not entirely α -helical. This type of C-terminal domain is often very long (> 70 aa) and is

680 found in the BetT_{Ec}, BetT_{Ps}, BetP_{Hi}, BetS and BetU transporters (Table 1). For several
681 members of the BCCT carriers, namely BetL, BetP_{Vp}, OpuD_{Vc}, and LcoP (Table 1), the
682 assignment to the three types of C-terminal domains is not a trivial task, since they all
683 comprise charged amino acid clusters together with flexible stretches of glycine and/or
684 proline residues. A BetP-like helix h7 and a BetP-like C-terminal domain are found together
685 only in the glycine betaine transporter ButA from *Tetragenococcus halophila* (Table 1)
686 (Baliarda *et al.*, 2003).

687 Interactions of C-terminal domains with the cytoplasmic side of adjacent subunits are
688 one of the main differences between the BetP (Ressl *et al.*, 2008) and the CaiT structures
689 (Tang *et al.*, 2010; Schulze *et al.*, 2010). The C-terminal interactions are not symmetrical in
690 the BetP trimer resulting in a break in non-crystallographic threefold symmetry. A break in
691 symmetry was also observed for BetP embedded in the membrane in 2D crystals (Ziegler *et al.*,
692 2004), while the CaiT trimer exhibits a perfect threefold symmetry. Unfortunately,
693 electron crystallographic data of CaiT are not available yet at the required resolution, but with
694 respect to trimer formation it is very likely that CaiT is also a symmetric trimer in the
695 membrane. In contrast, each subunit in the BetP trimer adopts a distinct conformation (Tsai *et al.*,
696 submitted). The different conformations of each subunit in membrane-embedded BetP,
697 the structural flexibility of the C-terminal domains and of the BetP trimer suggest a role of
698 trimerization far beyond protein stabilization in BetP. Together with the fact the C-terminal
699 domains acts as osmoregulator, the observed C-terminal interactions point towards a
700 functional role in transport activity regulation in BetP (Krämer, 2009). A possible scenario
701 would be that the subunits communicate their conformational state *via* the trimer interface to
702 synchronize their transport activity. Thereby, the BetP glycine betaine transporter would take
703 advantage of the trimeric state in a very elegant fashion.

704

705 **Sensing and signal transduction by BetP at the biochemical level**

706 A considerable number of the BCCT carriers listed in Table 1 respond to osmotic stress but
707 this issue has only been studied in detail for a few of these transporters. In view of the
708 multiplicity of potential physical stimuli directly or indirectly related to osmotic stress (Wood,
709 1999; Bremer and Krämer, 2000; Poolman *et al.*, 2004; Wood, 2006; Krämer, 2009;
710 Altendorf *et al.*, 2009), the analysis of the sensory input into a transporter protein is an
711 experimentally difficult task. For BetP, the situation was simplified by the fact that the
712 reconstituted protein in proteoliposomes was fully capable of responding to osmotic stress

713 (Rübenhagen *et al.*, 2000). Consequently, individual physical stimuli could be tested
714 separately and their relevance for carrier activation could be carefully evaluated.

715 The physical stimuli found to be relevant for the osmotic control of BetP activity can
716 been divided into two classes: (i) An increase in K^+ (and Rb^+ or Cs^+) triggers BetP activity but
717 only when applied at the cytoplasmic side (Rübenhagen *et al.*, 2001). The observed
718 specificity with respect to the activating agent practically excludes the unspecific effect of a
719 change in the degree of BetP hydration as a stimulus. An unspecific influence of osmolytes
720 and macromolecules has been proposed as a stimulus for osmotic control of the MFS-type
721 transporter ProP from *E. coli* (Culham *et al.*, 2003). (ii) BetP activity is also affected by
722 stimuli originating from the membrane. A modulating effect of local anaesthetics inserted into
723 the membrane affects BetP-mediated glycine betaine transport (Steger *et al.*, 2004). This is
724 not an unspecific effect, since these types of compounds did not affect the BCCT carrier EctP
725 from *C. glutamicum*. It was also observed that the surface charge density of the surrounding
726 membrane strongly modulates BetP activity; the concentration of K^+ necessary for BetP
727 activation increased in parallel to the content of negatively charged lipids (Schiller *et al.*,
728 2006). In addition to osmotic stress, BetP was also activated by chill stress (Özcan *et al.*,
729 2005). Since this effect was independent of changes in K^+ content the relevant stimulus should
730 originate from changes in the physical state of the membrane. Finally, membranes with
731 changed lipid composition but unchanged surface charge density exhibit altered regulation of
732 the BetP transporter (Özcan *et al.*, 2007). Taken together, signal input leading to stimulation
733 of BetP transporter activity seems to be of two types: a rise in the intracellular K^+
734 concentration that follows an osmotic up-shift and a stimulus originating from the membrane
735 into which the BetP transporter is embedded.

736 Mutational studies of BetP have provided substantial information on the contribution
737 of selected domains with respect to its transport function and regulation of its activity by high
738 osmolarity. A stepwise truncation of the C-terminal domain of BetP showed that the C-
739 terminal region containing the second charge cluster could be deleted without significant
740 impact on transporter function. Further truncation of the C-terminal domain by 25 amino acid
741 residues or more, including the first charge cluster, led to BetP transporter derivatives with
742 decreased but constitutive activity (Peter *et al.*, 1998a; Schiller *et al.*, 2004). Full removal of
743 the C-terminal domain rendered BetP inactive. Interestingly, activation of BetP activity at low
744 temperature, probably by a stimulus derived from the surrounding membrane, was not
745 influenced by a truncation of the C-terminal domain (Özcan *et al.*, 2005). The functional
746 significance of the C-terminal domain of BetP was further studied by amino acid scanning

747 mutagenesis (amino acid replacements with Ala or Pro residues) (Ott *et al.*, 2008). These
748 experiments led to two important conclusions: (i) a particular region in the middle of the C-
749 terminal domain of BetP was found to be critical for sensory function with respect to its
750 conformational properties rather than its charge distribution, and (ii) interaction of the C-
751 terminal domain with particular loops at the cytoplasmic face of BetP and with the N-terminal
752 domain was detected (Ott *et al.*, 2008). Consequently, both terminal domains, and in
753 particular the C-terminus, are critically involved in the activation of BetP by osmotic stress.
754 BetT-like choline transporter proteins have C-terminal domains that are substantially longer
755 than that present in BetP (Table 1). In both *E. coli* and *P. syringae*, BetT proteins with
756 truncated C-terminal domains gradually lose their capacity for osmotic activation, indicating
757 that these domains are involved in osmosensing and/or osmoregulation of transporter activity
758 (Tondervik and Strom, 2007; Chen and Beattie, 2008).

759

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

761 Biochemical data elucidated three key players for sensing and signal transduction by BetP: (i)
762 the C-terminal domain, (ii) cytoplasmic loops, and (iii) the N-terminal domain (Fig. 8). While
763 the N-terminal domain is not resolved in the X-ray structure of BetP (Ressl *et al.*, 2009),
764 biochemical data concerning the functional impact of cytoplasmic loops, especially of loop 2
765 and loop 8, and of the C-terminal domain on osmotic activation and transporter function can
766 be correlated to the BetP crystal structure. Loop 2 preceding TM3 contains a cluster of
767 negatively charged residues (Asp-131, Glu-132 and Glu-135). Mutations at Asp-131 and Glu-
768 132 somehow prevent expression of the *betP* structural gene and a mutation at Glu-135
769 reduces transport activity (Ott *et al.*, 2008). The BetP structure reveals an interaction of Asp-
770 131 via a salt bridge with an Arg residue located in the C-terminal helix of the adjacent
771 subunit, and Glu-132 with an Arg residue in loop 8, which connects TM8 and TM9 and is part
772 of the signature sequence of the BCCT family (Kappes *et al.*, 1996; Saier, 2000). Within this
773 network, changes in orientation of the C-terminal helix in one subunit of BetP would affect
774 the orientation and conformation of loop 2 and loop 8 in an adjacent subunit. It seems likely
775 that such interactions would have consequences for structural features of TM3 and TM8, both
776 of which are involved in substrate binding and Na⁺ coupling. Thus, the structure of BetP
777 suggests a “locking” interaction of the C-terminal domain with these loops, which might
778 translate into an inhibition of transport activity and, consequently, seems to be partly
779 responsible for regulating the transport cycle of BetP.

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

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

808 Subunit interactions within the trimer seem to be an essential feature of regulation of
809 transport activity in members of the BCCT carriers. This might explain some differences in
810 regulatory behaviour of osmoregulated BCCT carriers such as BetP compared to
811 osmoregulated transporters of other transporter families, e.g. ProP from *E. coli* (Culham *et al.*,
812 2008a; Culham *et al.*, 2008b) and OpuA from *L. lactis* (van der Heide and Poolman, 2000;
813 van der Heide *et al.*, 2001; Mahmood *et al.*, 2009). In fact, this idea has already been

814 suggested in a previous review (Poolman *et al.*, 2004). However, the concepts derived for the
815 osmotic activity control of transporters involved in compatible solute acquisition must be
816 viewed with caution, since the transporter families, the structure of the OpuA, ProP and BetP
817 transporters and their subunit composition are rather different.

818 Moreover, the putative primary trigger that controls transport activity in response to a
819 high osmolarity challenge seems to be rather different as well: a change in intracellular water
820 availability in the case of ProP (Culham *et al.*, 2003), a change in the intracellular ionic
821 strength for OpuA (van der Heide and Poolman, 2000; van der Heide *et al.*, 2001; Mahmood
822 *et al.*, 2009), and altered cytoplasmic K⁺ concentration in the case of BetP (Rübenhagen *et al.*,
823 2001). Nevertheless, some common features can be deduced from the biochemical analysis
824 the ProP, OpuA and BetP transport systems. Turgor was not found to be responsible for
825 activation of any of these transporters; a switching behaviour of terminal domains as a
826 mechanistic element in regulation has been suggested for all three; and an impact of
827 membrane conditions and composition on carrier activity was found to be relevant for ProP,
828 OpuA and BetP (van der Heide *et al.*, 2001; Poolman *et al.*, 2004; Schiller *et al.*, 2006;
829 Mahmood *et al.*, 2009; Romantsov *et al.*, 2009). It is not possible to generalise, however, on
830 the particular nature of the transport activity-controlling stimulus, the mechanistic details of
831 stimulus sensing, and the concept of how the transport cycle should be connected to the
832 regulatory cycle of the various transporters under study.

833

834 **The BCCT carriers: conclusions and perspectives**

835 When initially recognized in 1996, the BCCT family of transporters comprised just 4
836 functionally characterized members (Kappes *et al.*, 1996). Since then, the number of
837 functionally studied BCCT carriers has modestly risen to 22 (Table 1). The explosion in the
838 number of available genome sequences of microorganisms revealed a widespread occurrence
839 of BCCT carriers (currently about 2 200 representatives) that are predominantly present in
840 members of the *Bacteria*. Substrates for BCCT carriers different from those originally found
841 (Kappes *et al.*, 1996) have also been detected in the meantime (Fig. 2). Most notably are
842 transporters for ectoines and for DMSP.

843 BCCT carriers for ectoine and hydroxyectoine (Table 1), widely synthesized as
844 osmostress- and thermostress-protectants by members of the *Bacteria* (Garcia-Esteba *et al.*,
845 2006; Bursy *et al.*, 2007; Bursy *et al.*, 2008; Kuhlmann *et al.*, unpublished results), are of
846 interest from a crystallographic point of view because the chemical structures of these
847 compatible solutes differ considerably from those of other substrates for BCCT carriers (Fig.

848 2). Crystal structures of soluble ectoine and hydroxyectoine binding proteins operating in the
849 framework of either ABC- or TRAP-type transporters have recently been determined
850 (Hanekop *et al.*, 2007; Kuhlmann *et al.*, 2008; Lecher *et al.* 2009). It will be interesting to see
851 if, similar to the situation found for the BetP transporter and the periplasmic binding protein
852 ProX (Ressl *et al.*, 2009; Schiefner *et al.*, 2004a), the ligand-binding site present in
853 ectoine/hydroxyectoine-specific BCCT carriers will correspond to that of the aforementioned
854 soluble ectoine/hydroxyectoine binding proteins.

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

863 The crystal structures of Na⁺-coupled BetP transporter (Ressl *et al.*, 2009) and the
864 substrate:product antiporter CaiT (Tang *et al.*, 2010; Schulze *et al.*, 2010) provided the
865 opportunity to correlate structural features of functionally distinct members of the BCCT
866 family with a wealth of biochemical and physiological data (Jung *et al.*, 2002; Vinothkumar
867 *et al.*, 2006; Schiller *et al.*, 2006; Peter *et al.*, 1998a; Rübenhagen *et al.*, 2001; Ott *et al.*,
868 2008; Krämer, 2009). With respect to BetP, the combination of biochemical and
869 crystallographic analysis provided unprecedented insight into the functioning and activity
870 control of a secondary transporter responsive to osmotic stress. The regulatory response of
871 BetP to increased osmolarity was found to require a partly α -helical C-terminal domain of the
872 transporter and, furthermore, evidence has been provided that the trimeric state may be an
873 requirement for functional regulation. Based on the biochemical characteristics and crystal
874 structures of BetP and CaiT, we suggest that many BCCT carriers should form trimeric
875 complexes within the membrane. Our analysis of BetP and CaiT indicates that there might be
876 a relationship between the flexibility of the BetP-type trimer and the osmotic regulation of
877 transport activity and the rigidity of the CaiT-type trimer that is not able to regulate transport
878 activity in response to osmotic challenges. However, we note that structural determinants of
879 trimerization apparently show significant variation with the BCCT carriers. A combination of
880 X-ray crystallography and biochemical analysis will be required to further unravel substrate

881 binding, transport mechanism and activity regulation of additional members of the BCCT-
882 carriers in response to environmental stimuli.

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

888

889 **Acknowledgements**

890 Work in the author's laboratories on bacterial osmoregulation and the biochemical and
891 structural analysis of BCCT carriers was funded by grants from the Deutsche
892 Forschungsgemeinschaft (DFG), the Max-Planck Society, the Fonds der Chemischen
893 Industrie and the LOEWE program of the State of Hessen (SynMicro LOEWE Centre;
894 Marburg). We are grateful to Vickie Koogler for her kind help in editing of the manuscript.
895 We apologize to all our colleagues whose important work on microbial osmoadaptation and
896 compatible solute transporters could not be appropriately cited due to space limitations.

897

898

899

900 **References**

901 Abramson, J. and Wright, E. M. (2009) Structure and function of Na⁺-symporters with
902 inverted repeats. *Curr Opin Struc Biol* **19**: 425-432.

903 Altendorf, K., Booth, I. R., Gralla, J., Greie, J.-C., Rosenthal, A. Z. and Wood, J. M. (2009)
904 Chapter 5.4.5, Osmotic Stress. In: *EcoSal-Escherichia coli and Salmonella: cellular*
905 *and molecular biology*. A. Böck *et. al.* (eds). Washington: ASM Press.

906 Angelidis, A. S. and Smith, G. M. (2003) Role of the glycine betaine and carnitine
907 transporters in adaptation of *Listeria monocytogenes* to chill stress in defined medium.
908 *Appl Environ Microbiol* **69**: 7492-7498.

909 Anishkin, A. and Sukharev, S. (2009) State-stabilizing interactions in bacterial
910 mechanosensitive channel gating and adaptation. *J Biol Chem* **284**: 19153-19157.

911 Arakawa, T. and Timasheff, S. N., (1985) The stabilization of proteins by osmolytes.
912 *Biochem J* **47**: 411-414.

- 913 Auton, M., Bolen, W.D. and Rösgen, J. (2008) Structural thermodynamics of protein
914 preferential solvation: osmolyte solvation of proteins, aminoacids, and peptides.
915 *Proteins* **73**: 802-813.
- 916 Baliarda, A., Robert, H., Jebbar, M., Blanco, C. and Le Marrec, C. (2003) Isolation and
917 characterization of ButA, a secondary glycine betaine transport system operating in
918 *Tetragenococcus halophila*. *Curr Microbiol* **47**: 347-351.
- 919 Bolen, D. W. and Baskakov, I. V. (2001) The osmophobic effect: natural selection of a
920 thermodynamic force in protein folding. *J Mol Biol* **310**: 955-963.
- 921 Bolen, D. W. and Rose, G. D. (2008) Structure and energetics of the hydrogen-bonded
922 backbone in protein folding. *Annu Rev Biochem* **77**: 339-362.
- 923 Booth, I. R., Edwards, M. D., Black, S., Schumann, U. and Miller, S. (2007)
924 Mechanosensitive channels in bacteria: signs of closure? *Nat Rev Microbiol* **5**: 431-
925 440.
- 926 Bremer, E. (2002) Adaptation to changing osmolarity. In: *Bacillus subtilis* and its closest
927 relatives: from genes to cells. A. L. Sonenshein, J. A. Hoch & R. Losick (eds).
928 Washington: ASM Press, pp. 385-391.
- 929 Bremer, E. and Krämer, R. (2000) Coping with osmotic challenges: osmoregulation through
930 accumulation and release of compatible solutes. In: *Bacterial Stress Responses*. G.
931 Storz & R. Hengge-Aronis (eds). ASM Press, pp. 79-97.
- 932 Buchet, A., Eichler, K. and Mandrand-Berthelot, M. A. (1998) Regulation of the carnitine
933 pathway in *Escherichia coli*: investigation of the *cai-fix* divergent promoter region. *J*
934 *Bacteriol* **180**: 2599-2608.
- 935 Burg, M. B. and Ferraris, J. D. (2008) Intracellular organic osmolytes: function and
936 regulation. *J Biol Chem* **283**: 7309-7313.
- 937 Bursy, J., Pierik, A. J., Pica, N. and Bremer, E. (2007) Osmotically induced synthesis of the
938 compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine
939 hydroxylase. *J Biol Chem* **282**: 31147-31155.
- 940 Bursy, J., Kuhlmann, A. U., Pittelkow, M., Hartmann, H., Jebbar, M., Pierik, A.J. and
941 Bremer, E. (2008) Synthesis and uptake of the compatible solutes ectoine and 5-
942 hydroxyectoine by *Streptomyces coelicolor* A3(2) in response to salt and heat stress.
943 *Appl Environ Microbiol* **74**: 7286-7296.
- 944 Caldas, T., Demont-Caulet, N., Ghazi, A. and Richarme, G. (1999) Thermoprotection by
945 glycine betaine and choline. *Microbiology* **145** 2543-2548.

- 946 Caplan, D. A., Subbotina, J. O. and Noskov, S. Y., (2008) Molecular mechanism of ion-ion
947 and ion-substrate coupling in the Na⁺-dependent leucine transporter LeuT. *Biophys J*
948 **95**: 4613-4621.
- 949 Capp, M. W., Pegram, L. M., Saecker, R. M., Kratz, M., Riccardi, D., Wendorff, T., Cannon,
950 J. G. and Record, M. T., Jr. (2009) Interactions of the osmolyte glycine betaine with
951 molecular surfaces in water: thermodynamics, structural interpretation, and prediction
952 of m-values. *Biochemistry* **48**: 10372-103729.
- 953 Cayley, S. and Record, M. T., Jr. (2003) Roles of cytoplasmic osmolytes, water, and
954 crowding in the response of *Escherichia coli* to osmotic stress: biophysical basis of
955 osmoprotection by glycine betaine. *Biochemistry* **42**: 12596-12609.
- 956 Chen, C. and Beattie, G. A. (2008) *Pseudomonas syringae* BetT is a low-affinity choline
957 transporter that is responsible for superior osmoprotection by choline over glycine
958 betaine. *J Bacteriol* **190**: 2717-2725.
- 959 Cheung, K. J., Ba darinarayana, V., Selinger, D. W., Janse, D. and Church, G. M. (2003) A
960 microarray-based antibiotic screen identifies a regulatoty role for supercoiling in the
961 osmotic stress response of *Escherichia coli*. *Genome Res* **13**: 206-215.
- 962 Culham, D. E., Henderson, J., Crane, R. A. and Wood, J. M. (2003) Osmosensor ProP of
963 *Escherichia coli* responds to the concentration, chemistry, and molecular size of
964 osmolytes in the proteoliposome lumen. *Biochemistry* **42**: 410-420.
- 965 Culham, D. E., Romantsov, T. and Wood, J. M. (2008a) Roles of K⁺, H⁺, H₂O, and DeltaPsi
966 in solute transport mediated by major facilitator superfamily members ProP and LacY.
967 *Biochemistry* **47**: 8176-8185.
- 968 Culham, D. E., Vernikovska, Y., Tschowri, N., Keates, R. A., Wood, J. M. and Boggs, J. M.
969 (2008b) Periplasmic loops of osmosensory transporter ProP in *Escherichia coli* are
970 sensitive to osmolality. *Biochemistry* **47**: 13584-13593.
- 971 Dickschat, J. S., Zell, C. and Brock, N. (2010) Pathways and substrate specificity of DMSP
972 catabolism in marine bacteria of the *Roseobacter* clade. *ChemBioChem* **11**: 417-425.
- 973 Eichler, K., Bourgis, F., Buchet, A., Kleber, H. P. and Mandrand-Berthelot, M. A. (1994)
974 Molecular characterization of the *cai* operon necessary for carnitine metabolism in
975 *Escherichia coli*. *Mol Microbiol* **13**: 775-786.
- 976 Empadinhas, N. and da Costa, M. S. (2008) Osmoadaptation mechanisms in prokaryotes:
977 distribution of compatible solutes. *Int Microbiol* **11**: 151-161.
- 978 Faham, S. (2008) The crystal structure of a sodium galactose transporter reveals mechanistic
979 insights into Na⁺/sugar symport. *Science* **321**: 810-814.

- 980 Farwick, M., Siewe, R. M. and Krämer, R. (1995) Glycine betaine uptake after hyperosmotic
981 shift in *Corynebacterium glutamicum*. *J Bacteriol* **177**: 4690-4695.
- 982 Garcia-Esteva, R., Aragona, M., Reina-Bueno, M., Captote, N., Iglesia-Guerra, F., Nieto, J.J.
983 and Vragas, C. (2006) The *ectD* gene, which is involved in the synthesis of the
984 compatible solute hydroxyectoine, is essential for thermoprotection of the halophilic
985 bacterium *Chromohalobacter salexigens*. *J. Bacteriol.* **188**: 3774-3784.
- 986 Gouaux, E. (2009) The molecular logic of sodium-coupled neurotransmitter transporters.
987 *Phil. Trans. R. Soc. B* **364**: 149-154.
- 988 Grammann, K., Volke, A. and Kunte, H. J. (2002) New type of osmoregulated solute
989 transporter identified in halophilic members of the bacteria domain: TRAP transporter
990 TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM
991 2581(T). *J Bacteriol* **184**: 3078-3085.
- 992 Haardt, M., Kempf, B., Faatz, E. and Bremer, E. (1995) The osmoprotectant proline betaine is
993 a major substrate for the binding-protein-dependent transport system ProU of
994 *Escherichia coli* K-12. *Mol Gen Genet* **246**: 783-786.
- 995 Hagemann, M. (2010) Molecular biology of cyanobacterial salt acclimation. *FEMS Microbiol*
996 *Rev* (in press)
- 997 Hanekop, N., Höing, M., Sohn-Bösser, L., Jebbar, M., Schmitt, L. and Bremer, E. (2007)
998 Crystal structure of the ligand-binding protein EhuB from *Sinorhizobium meliloti*
999 reveals substrate recognition of the compatible solutes ectoine and hydroxyectoine. *J*
1000 *Mol Biol* **374**: 1237-1250.
- 1001 Holtmann, G. and Bremer, E. (2004) Thermoprotection of *Bacillus subtilis* by exogenously
1002 provided glycine betaine and structurally related compatible solutes: involvement of
1003 Opu transporters. *J Bacteriol* **186**: 1683-1693.
- 1004 Horn, C., Sohn-Bösser, L., Breed, J., Welte, W., Schmitt, L. and Bremer, E. (2006) Molecular
1005 determinants for substrate specificity of the ligand-binding protein OpuAC from
1006 *Bacillus subtilis* for the compatible solutes glycine betaine and proline betaine. *J Mol*
1007 *Biol* **357**: 592-606.
- 1008 Ignatova, Z. and Gierasch, L. M. (2006) Inhibition of protein aggregation in vitro and in vivo
1009 by a natural osmoprotectant. *Proc Natl Acad Sci USA* **103**: 13357-13361.
- 1010 Jardetzky, O. (1966) Simple allosteric model for membrane pumps. *Nature* **211**: 969-970.
- 1011 Jung, H., Buchholz, M., Clausen, J., Nietschke, M., Revermann, A., Schmid, R. and Jung, K.
1012 (2002) CaiT of *Escherichia coli*, a new transporter catalyzing L-carnitine/gamma-
1013 butyrobetaine exchange. *J Biol Chem* **277**: 39251-39258.

- 1014 Kappes, R. M., Kempf, B. and Bremer, E. (1996) Three transport systems for the
1015 osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD.
1016 *J Bacteriol* **178**: 5071-5079.
- 1017 Kempf, B. and Bremer, E. (1998) Uptake and synthesis of compatible solutes as microbial
1018 stress responses to high-osmolality environments. *Arch Microbiol* **170**: 319-330.
- 1019 Krämer, R. (2009) Osmosensing and osmosignaling in *Corynebacterium glutamicum*. *Amino*
1020 *Acids* **37**: 487-497.
- 1021 Krämer, R. (2010) Bacterial stimulus perception and signal transduction: response to osmotic
1022 stress. *Chem Rec* (in press).
- 1023 Krishnamurthy, H., Piscitelli, C. L. and Gouaux, E. (2009) Unlocking the molecular secrets of
1024 sodium-coupled transporters. *Nature* **459**: 347-355.
- 1025 Kuhlmann, S. I., Terwisscha van Scheltinga, A. C., Bienert, R., Kunte, H. J. and Ziegler, C.
1026 (2008) Osmoregulated transport of compatible solutes in the halophilic bacterium
1027 *Halomonas elongata*: 1.55 Å high-resolution structure of the periplasmic ectoine-
1028 binding protein from TRAP-transporter TeaABC. *Biochemistry* **47**: 9475-9485.
- 1029 Laloknam, S., Tanaka, K., Buabooch, T., Waditte, R., Incharoensakdi, A., Hibino, T., Tanaka,
1030 T., and Tkabe, T. (2006) Halotolerant cyanobacterium *Aphanothece halophytica*
1031 contains a betaine transporter active at alkaline pH and high salinity. *Appl Environ*
1032 *Microbiol* **72**: 6018-6026.
- 1033 Lamark, T., Kaasen, I., Eshoo, M. W., Falkenberg, P., McDougall, J. and Strom, A. R. (1991)
1034 DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-
1035 glycine betaine pathway of *Escherichia coli*. *Mol Microbiol* **5**: 1049-1064.
- 1036 Lamark, T., Rokenes, T. P., McDougall, J. and Strom, A. R. (1996) The complex *bet*
1037 promoters of *Escherichia coli*: regulation by oxygen (ArcA), choline (BetI), and
1038 osmotic stress. *J Bacteriol* **178**: 1655-1662.
- 1039 Lamark, T., Styrvold, O. B. and Strom, A. R. (1992) Efflux of choline and glycine betaine
1040 from osmoregulating cells of *Escherichia coli*. *FEMS Microbiol Lett* **75**: 149-154.
- 1041 Lecher, J., Pittelkow, M., Zobel, S., Bursy, J., Bönig, T., Smits, S. H. J., Schmitt, L. and
1042 Bremer, E. (2009) The crystal structure of UehA in complex with Ectoine-A
1043 comparison with Other TRAP-T binding proteins. *J Mol Biol* **389**: 58-73.
- 1044 Lolkema, J. S. and Slotboom, D. J. (2008) The major amino acid transporter superfamily has
1045 a similar core structure as Na⁺-galactose and Na⁺-leucine transporters. *Mol Membr*
1046 *Biol* **25**: 567-570.

- 1047 Ly, A., Henderson, J., Lu, A., Culham, D. E. and Wood, J. M. (2004) Osmoregulatory
1048 systems of *Escherichia coli*: identification of betaine-carnitine-choline transporter
1049 family member BetU and distributions of *betU* and *trkG* among pathogenic and
1050 nonpathogenic isolates. *J Bacteriol* **186**: 296-306.
- 1051 Mahmood, N. A., Biemans-Oldehinkel, E. and Poolman, B. (2009) Engineering of ion
1052 sensing by the cystathionine beta-synthase module of the ABC transporter OpuA. *The*
1053 *J Biol Chem* **284**: 14368-14376.
- 1054 Martinac, B., Saimi, Y. and Kung, C. (2008) Ion channels in microbes. *Physiol Rev* **88**: 1449-
1055 1490.
- 1056 Mikkat, S. and Hagemann, M. (2000) Molecular analysis of the *ggtBCD* gene cluster of
1057 *Synechocystis* sp. strain PCC6803 encoding subunits of an ABC transporter for
1058 osmoprotective compounds. *Arch Microbiol* **174**: 273-282.
- 1059 Möker, N., Brocker, M., Schaffer, S., Kramer, R., Morbach, S. and Bott, M. (2004) Deletion
1060 of the genes encoding the MtrA-MtrB two-component system of *Corynebacterium*
1061 *glutamicum* has a strong influence on cell morphology, antibiotics susceptibility and
1062 expression of genes involved in osmoprotection. *Mol Microbiol* **54**: 420-438.
- 1063 Möker, N., Kramer, J., Uden, G., Krämer, R. and Morbach, S. (2007) In vitro analysis of the
1064 two-component system MtrB-MtrA from *Corynebacterium glutamicum*. *J Bacteriol*
1065 **189**: 3645-36459.
- 1066 Nagarajavel, V., Madhusudan, S., Dole, S., Rahmouni, A. R. and Schnetz, K. (2007)
1067 Repression by binding of H-NA within the transcription unit. *J. Biol Chem* **282**:
1068 23622-23630.
- 1069 Noskov, S. Y. and Roux, B. (2008) Control of ion selectivity in LeuT: two Na⁺ binding sites
1070 with two different mechanisms. *J Mol Biol* **377**: 804-818.
- 1071 Oren, A. (1999) Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63**: 334-348.
- 1072 Oren, A. (2008) Microbial life at high salt concentrations: phylogenetic and metabolic
1073 diversity. *Saline Systems* **4**: 2.
- 1074 Oswald, C., Smits, S. H., Hoing, M., Sohn-Bosser, L., Dupont, L., Le Rudulier, D., Schmitt,
1075 L. and Bremer, E. (2008) Crystal structures of the choline/acetylcholine substrate-
1076 binding protein ChoX from *Sinorhizobium meliloti* in the liganded and unliganded-
1077 closed states. *J Biol Chem* **283**: 32848-32859.
- 1078 Ott, V., Koch, J., Späte, K., Morbach, S. and Krämer, R. (2008) Regulatory properties and
1079 interaction of the C- and N-terminal domains of BetP, an osmoregulated betaine
1080 transporter from *Corynebacterium glutamicum*. *Biochemistry* **47**: 12208-12218.

- 1081 Özcan, N., Ejsing, C. S., Shevchenko, A., Lipski, A., Morbach, S. and Krämer, R. (2007)
1082 Osmolality, temperature, and membrane lipid composition modulate the activity of
1083 betaine transporter BetP in *Corynebacterium glutamicum*. *J Bacteriol* **189**: 7485-7496.
- 1084 Özcan, N., Krämer, R. and Morbach, S. (2005) Chill activation of compatible solute
1085 transporters in *Corynebacterium glutamicum* at the level of transport activity. *J*
1086 *Bacteriol* **187**: 4752-4759.
- 1087 Padan, E., Bibi, E., Ito, M. and Krulwich, T.A. (2005) Alkaline pH homeostasis in bacteria:
1088 new insights. *Biochim Biohys Acta* **1717**: 67-88.
- 1089 Pichereau, V., Pocard, J-A., Hamelin, J., Blanco, C. and Bernard, T. (1998) Differential
1090 effects of dimethylsulfoniopropionate, dimethylsulfonioacetate, and other S-
1091 methylated compounds on the growth of *Sinorhizobium meliloti* at low and high
1092 osmolarities. *Appl Environ Microbiol* **64**: 1420-1429.
- 1093 Perozo, E. (2006) Gating prokaryotic mechanosensitive channels. *Nat Rev Mol Cell Biol* **7**:
1094 109-119.
- 1095 Peter, H., Burkovski, A. and Krämer, R. (1996) Isolation, characterization, and expression of
1096 the *Corynebacterium glutamicum betP* gene, encoding the transport system for the
1097 compatible solute glycine betaine. *J Bacteriol* **178**: 5229-5234.
- 1098 Peter, H., Burkovski, A. and Kramer, R. (1998a) Osmo-sensing by N- and C-terminal
1099 extensions of the glycine betaine uptake system BetP of *Corynebacterium*
1100 *glutamicum*. *J Biol Chem* **273**: 2567-2574.
- 1101 Peter, H., Weil, B., Burkovski, A., Krämer, R. and Morbach, S. (1998b) *Corynebacterium*
1102 *glutamicum* is equipped with four secondary carriers for compatible solutes:
1103 identification, sequencing, and characterization of the proline/ectoine uptake system,
1104 ProP, and the ectoine/proline/glycine betaine carrier, EctP. *J Bacteriol* **180**: 6005-
1105 6012.
- 1106 Poolman, B., Blount, P., Folgering, J. H., Friesen, R. H., Moe, P. C. and van der Heide, T.
1107 (2002) How do membrane proteins sense water stress? *Mol Microbiol* **44**: 889-902.
- 1108 Poolman, B. and Glaasker, E. (1998) Regulation of compatible solute accumulation in
1109 bacteria. *Mol Microbiol* **29**: 397-407.
- 1110 Poolman, B., Spitzer, J. J. and Wood, J. M. (2004) Bacterial osmosensing: roles of membrane
1111 structure and electrostatics in lipid-protein and protein-protein interactions. *Biochim*
1112 *Biophys Acta* **1666**: 88-104.

- 1113 Record, M. T., Jr., Courtenay, E. S., Cayley, D. S. and Guttman, H. J. (1998) Responses of *E.*
1114 *coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water.
1115 *TIBS* **23**: 143-148.
- 1116 Ressler, S., Terwisscha van Scheltinga, A. C., Vonnheim, C., Ott, V. and Ziegler, C. (2009)
1117 Molecular basis of transport and regulation in the Na⁺/betaine symporter BetP. *Nature*
1118 **458**: 47-52.
- 1119 Romantsov, T., Guan, Z. and Wood, J. M. (2009) Cardiolipin and the osmotic stress
1120 responses of bacteria. *Biochim Biophys Acta* **1788**: 2092-2100.
- 1121 Romeo, Y., Bouvier, J. and Gutierrez, C. (2007) Osmotic regulation of transcription in
1122 *Lactococcus lactis*: ionic-strength-dependent binding of the BusR repressor to the *busA*
1123 promoter. *FEBS Lett.* **581**: 3387-3390.
- 1124 Rosenstein, R., Futter-Bryniok, D. and Götz, F. (1999) The choline-converting pathway in
1125 *Staphylococcus xylosum* C2A: genetic and physiological characterization. *J Bacteriol*
1126 **181**: 2273-2278.
- 1127 Rosenthal, A. Z., Kim, Y. and Gralla, J. D. (2008) Poising of the *Escherichia coli* RNA
1128 polymerase and its release from the sigma 38 C-terminal tail for *osmY* transcription. *J*
1129 *Mol Biol* **376**: 938-949.
- 1130 Rösgen, J. (2007) Molecular basis of osmolyte effects on protein and metabolites. *Methods in*
1131 *Enzymol* **428**: 459-486.
- 1132 Rösgen, J. (2009) Molecular crowding and solvation: direct and indirect impact on protein
1133 reactions. *Methods in molecular biology (Clifton, N.J)* **490**: 195-225.
- 1134 Rübenhagen, R., Morbach, S. and Krämer, R. (2001) The osmoreactive betaine carrier BetP
1135 from *Corynebacterium glutamicum* is a sensor for cytoplasmic K⁺. *EMBO J* **20**: 5412-
1136 5420.
- 1137 Rübenhagen, R., Rönsch, H., Jung, H., Krämer, R. and Morbach, S. (2000) Osmosensor and
1138 osmoregulator properties of the betaine carrier BetP from *Corynebacterium*
1139 *glutamicum* in proteoliposomes. *J Biol Chem* **275**: 735-741.
- 1140 Saier, M. H., Jr. (2000) A functional-phylogenetic classification system for transmembrane
1141 solute transporters. *Microbiol Mol Biol Rev* **64**: 354-411.
- 1142 Saier, M. H., Jr., Yen, M. R., Noto, K., Tamang, D. G. and Elkan, C. (2009) The transporter
1143 classification database: recent advances. *Nucleic Acids Res* **37**: D274-278..
- 1144 Schiefner, A., Breed, J., Bosser, L., Kneip, S., Gade, J., Holtmann, G., Diederichs, K., Welte,
1145 W. and Bremer, E. (2004a) Cation- π interactions as determinants for binding of the

- 1146 compatible solutes glycine betaine and proline betaine by the periplasmic ligand-
1147 binding protein ProX from *Escherichia coli*. *J Biol Chem* **279**: 5588-5596.
- 1148 Schiefner, A., Holtmann, G., Diederichs, K., Welte, W. and Bremer, E. (2004b) Structural
1149 basis for the binding of compatible solutes by ProX from the hyperthermophilic
1150 archaeon *Archaeoglobus fulgidus*. *J Biol Chem* **279**: 48270-48281.
- 1151 Schiller, D., Ott, V., Krämer, R. and Morbach, S. (2006) Influence of membrane composition
1152 on osmosensing by the betaine carrier BetP from *Corynebacterium glutamicum*. *The J*
1153 *Biol Chem* **281**: 7737-7746.
- 1154 Schiller, D., Rübenhagen, R., Krämer, R. and Morbach, S. (2004) The C-terminal domain of
1155 the betaine carrier BetP of *Corynebacterium glutamicum* is directly involved in
1156 sensing K⁺ as an osmotic stimulus. *Biochemistry* **43**: 5583-5591.
- 1157 Schulze, S., Köster, S., Geldmacher, U., Terwisscha van Scheltinga, A. C. and Kühlbrandt,
1158 W. (2010) Structural basis of Na⁺-independent and cooperative substrate/product
1159 antiport in CaiT. *Nature* (in press).
- 1160 Shaffer, P. L., Goehring, A., Shankaranarayanan, A. and Gouaux, E. (2009) Structure and
1161 mechanism of a Na⁺-independent amino acid transporter. *Science* **325**: 1010-1014.
- 1162 Sheehan, V. M., Sleator, R. D., Fitzgerald, G. F. and Hill, C. (2006) Heterologous expression
1163 of BetL, a betaine uptake system, enhances the stress tolerance of *Lactobacillus*
1164 *salivarius* UCC118. *Appl Environ Microbiol* **72**: 2170-2177.
- 1165 Singh, S., Yamashita, A. and Gouaux, E. (2007) Antidepressant binding site in a bacterial
1166 homologue of neurotransmitter transporters. *Nature* **448**: 952-956.
- 1167 Singh, S. K., Piscitelli, C. L., Yamashita, A. and Gouaux, E. (2008) A competitive inhibitor
1168 traps LeuT in an open-to-out conformation. *Science* **322**: 1655-1661.
- 1169 Smits, S. H., Hoing, M., Lecher, J., Jebbar, M., Schmitt, L. and Bremer, E. (2008) The
1170 compatible-solute-binding protein OpuAC from *Bacillus subtilis*: ligand binding, site-
1171 directed mutagenesis, and crystallographic studies. *J Bacteriol* **190**: 5663-5671.
- 1172 Spiegelhalter, F. and Bremer, E. (1998) Osmoregulation of the *opuE* proline transport gene
1173 from *Bacillus subtilis* - contributions of the σ^A - and σ^B -dependent stress-responsive
1174 promoters. *Mol Microbiol* **29**: 285-296.
- 1175 Steger, R., Weinand, M., Krämer, R. and Morbach, S. (2004) LcoP, an osmoregulated
1176 betaine/ectoine uptake system from *Corynebacterium glutamicum*. *FEBS Lett* **573**:
1177 155-160.
- 1178 Street, T. O., Bolen, D. W. and Rose, G. D. (2006) A molecular mechanism for osmolyte-
1179 induced protein stability. *Proc Natl Acad Sci U S A* **103**: 13997-14002.

- 1180 Street, T. O., Krukenberg, K. A., Rosgen, J., Bolen, D. W. and Agard, D. A. (2010)
1181 Osmolyte-induced conformational changes in the Hsp90 molecular chaperone. *Protein*
1182 *Sci* **19**: 57-65.
- 1183 Tang, L., Bai, L., Wang, W-h and Jiang, T. (2010) Crystal structure of the carnitine
1184 transporter and insights into the antiport mechanism. *Natr Struct Mol Biol* **17**: 492-
1185 496.
- 1186 Tanghe, A., Van Dijck, P. and Thevelein, J. M. (2006) Why do microorganisms have
1187 aquaporins? *Trends Microbiol* **14**: 78-85.
- 1188 Todd, J. D., Curson, A. R., Nikolaidou-Katsaraidou, N., Brearley, C. A., Watmough, N. J.,
1189 Chan, Y., Page, P. C., Sun, L. and Johnston, A. W. (2010) Molecular dissection of
1190 bacterial acrylate catabolism - unexpected links with dimethylsulfoniopropionate
1191 catabolism and dimethyl sulfide production. *Environ Microbiol* **21**: 327-343.
- 1192 Tondervik, A. and Strom, A. R. (2007) Membrane topology and mutational analysis of the
1193 osmotically activated BetT choline transporter of *Escherichia coli*. *Microbiology* **153**:
1194 803-813.
- 1195 van der Heide, T. and Poolman, B. (2000) Osmoregulated ABC-transport system of
1196 *Lactococcus lactis* senses water stress via changes in the physical state of the
1197 membrane. *Proc Natl Acad Sci U S A* **97**: 7102-7106.
- 1198 van der Heide, T., Stuart, M. C. and Poolman, B. (2001) On the osmotic signal and
1199 osmosensing mechanism of an ABC transport system for glycine betaine. *EMBO J* **20**:
1200 7022-7032.
- 1201 Vial-Costa, M., Rinta-Kato, J. M., Sun, S., Sharma, S., Potestky, R. and Moran, M.A. (2010)
1202 Transcriptomic analysis of a bacterial community enriched with
1203 dimethylsulfoniopropionate. *ISME J* (in press).
- 1204 Vinothkumar, K. R., Raunser, S., Jung, H. and Kuhlbrandt, W. (2006) Oligomeric structure of
1205 the carnitine transporter CaiT from *Escherichia coli*. *J Biol Chem* **281**: 4795-4801.
- 1206 Welsh, D. T. (2000) Ecological significance of compatible solute accumulation by micro-
1207 organisms: from single cells to global climate. *FEMS Microbiol Rev* **24**: 263-290.
- 1208 Weyand, S. (2008) Structure and molecular mechanism of a nucleobase-cation-symport-1
1209 family transporter. *Science* **322**: 709-713.
- 1210 Wolters, J.C., Berntsson, R.P., Gul, N., Karasawa, A., Thunnissen, A.M., Slotboom, D.J. and
1211 Poolman, B. (2010) Ligand binding and crystal structures of the substrate-binding
1212 domain of the ABC transporter OpuA. *PLoS One* **5**: e10361

- 1213 Wood, J. M. (1999) Osmosensing by bacteria: signals and membrane-based sensors.
1214 *Microbiol Mol Biol Rev* **63**: 230-262.
- 1215 Wood, J. M. (2006) Osmosensing by bacteria. *Sci STKE* **357**: pe 43.
- 1216 Wood, J. M., Bremer, E., Csonka, L. N., Krämer, R., Poolman, B., van der Heide, T. and
1217 Smith, L. T. (2001) Osmosensing and osmoregulatory compatible solute accumulation
1218 by bacteria. *Comp Biochem Physiol A Mol Integr Physiol* **130**: 437-460.
- 1219 Yamashita, A., Singh, S. K., Kawate, T., Jin, Y. and Gouaux, E. (2005) Crystal structure of a
1220 bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters. *Nature* **437**:
1221 215-223.
- 1222 Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982) Living
1223 with water stress: the evolution of osmolyte systems. *Science* **217**: 1214-1222.
- 1224 Yancey, P. H. (2005) Organic osmolytes as compatible, metabolic and counteracting
1225 cytoprotectants in high osmolarity and other stresses. *J Exp Biol* **208**: 2819-2830.
- 1226 Zhou, Z. (2007) LeuT-desipramine structure reveals how antidepressants block
1227 neurotransmitter uptake. *Science* **317**: 1390-1393.
- 1228 Ziegler, C., Morbach, S., Schiller, D., Kramer, R., Tziatzios, C., Schubert, D. and Kuhlbrandt,
1229 W. (2004) Projection structure and oligomeric state of the osmoregulated
1230 sodium/glycine betaine symporter BetP of *Corynebacterium glutamicum*. *J Mol Biol*
1231 **337**: 1137-1147.

1232

1233 **Legends to Figures:**

1234

1235 **Fig. 1.** Osmotically driven water fluxes across the cytoplasmic membrane of a microbial cell
1236 exposed either to hyper- or hypo-osmotic surroundings.

1237

1238 **Fig. 2.** Known substrates for BCCT carriers. The positively charged nitrogen or sulfur head-
1239 groups and the delocalized positive charge present in ectoine and hydroxyectoine are
1240 highlighted.

1241

1242 **Fig. 3.** Activation profile of the BetP transporter embedded in different membranes. The rate
1243 of glycine betaine uptake was measured as a function of the osmolarity of the surrounding
1244 medium, either in the native membrane of *C. glutamicum* (red line), after heterologous
1245 expression in *E. coli* (green line), or after isolation, purification and reconstitution into
1246 proteoliposomes (blue line). The maximum activity measured was set to 100%, the absolute

1247 values are in the range of 30 – 80 $\mu\text{moles}/(\text{min} \cdot \text{mg dw})^{-1}$ for cells and about 1000 - 2000
1248 $\mu\text{moles}/(\text{min} \cdot \text{mg protein})^{-1}$ for proteoliposomes.

1249

1250 **Fig. 4.** Schematic representation of the catalytic and the activation (regulatory) cycle of the
1251 BetP transporter. In the activation cycle, BetP (C = carrier) occurs either in the active (C*,
1252 blue and yellow colour) or the inactive state (C, red colour). In the catalytic cycle, BetP
1253 exposes its glycine betaine substrate-binding site either to the periplasmic space (C*_e, carrier
1254 protein symbol open to the top) or to the cytoplasmic space (C*_i, carrier protein symbol open
1255 to the bottom). Alternatively, the substrate-binding site is occluded (yellow colour) from the
1256 surroundings. Substrate (S) denotes one molecule of glycine betaine (B; black triangle)
1257 together with 2 Na⁺ ions (green circles). In the course of one round of the catalytic cycle of
1258 the BetP carrier, one molecule of glycine betaine and two Na⁺ ions are translocated across the
1259 plasma membrane.

1260

1261 **Fig. 5.** Crystal structure of BetP from *C. glutamicum* and CaiT from *E. coli* in complex with
1262 their ligands. (A) Periplasmic view on the BetP trimer (resolved to a resolution of 3.35Å) in
1263 spiral presentation and on the CaiT trimer (resolved to a resolution of 3.5Å). Individual
1264 subunits are coloured in blue, red and green (B) Side view on the BetP and CaiT trimers. The
1265 C-terminal domain of subunit 1 from the BetP trimer assembly is shown to protrude 35 Å into
1266 the cytoplasm.

1267

1268 **Fig. 6. Topological organization of BetP within the plain of the cytoplasmic membrane.**
1269 (A) Top view on a BetP subunit with a colouring of the TM helices assigned according to
1270 BetP's topology (B) showing the organization of the two structural repeats.

1271

1272 **Fig. 7. Structural features and ligand binding sites of BetP and CaiT.** (A) Side view on
1273 the *C. glutamicum* BetP structure in complex with one molecule of glycine betaine; the CaiT
1274 structure from *E. coli* in complex with four L-carnitine molecules and the CaiT structure from
1275 *P. mirabilis* in complex with two γ -butyrobetaine molecules. (B) Structural features of the
1276 central ligand-binding site in BetP and CaiT. The aromatic ligand-binding boxes coordinating
1277 the substrate glycine betaine, L-carnitine or γ -butyrobetaine are highlighted. (C) Cross-
1278 section through the BetP and CaiT structures revealing an occluded state for BetP and an
1279 inward-facing state for CaiT, respectively.

1280

1281 **Fig. 8.** Osmoregulatory interactions within the BetP trimer. Spatial interaction of the C-
1282 terminal domain of one subunit (monomer 1) of BetP with charged residues in loop 2 and
1283 loop 8 of the adjacent BetP subunit (monomer 3), which is suggested to be of significance for
1284 activity regulatory. (A) Under low external osmolarity and/or absence of K^+ ions (inactive
1285 conditions) the BetP carrier is locked in a resting conformation with the bound two Na^+ ions
1286 (green circles) and glycine betaine (B; black triangle) occluded from the cytoplasmic side. (B)
1287 Under hyperosmotic conditions and increasing concentrations of K^+ ions (grey spheres) in the
1288 cytoplasm and stimuli perceived via the membrane, the BetP carrier initiates a
1289 orientation/conformation change of its C-terminal domain which alters the interaction
1290 network and allows a conformational change of the TM helices of the inverted repeats. As a
1291 consequence, glycine betaine can be released into the cytoplasm. The role of the N-terminal
1292 domain of BetP (monomer 3) in this interaction switching is currently not firmly understood.
1293 It is suggested that the negative charge clusters in BetP (red spheres) interact with the positive
1294 charge clusters (blue spheres) present the C-terminal domain of BetP (picture kindly provided
1295 by Susanne Ressler).

1296

1297

1298

1299

1300

1301

1302

1303

1304

1305

1306

1307

1308

1309

1310

1311

1312

1313

1314

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]	<i>Corynebacterium glutamicum</i> CAA63771.1	GB	smf / symport	595	100	58	50

1315

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

1316 **Table 1.** Functionally characterized members of the BCCT family

1317

1318

1319

1320 **References for Table 1:**

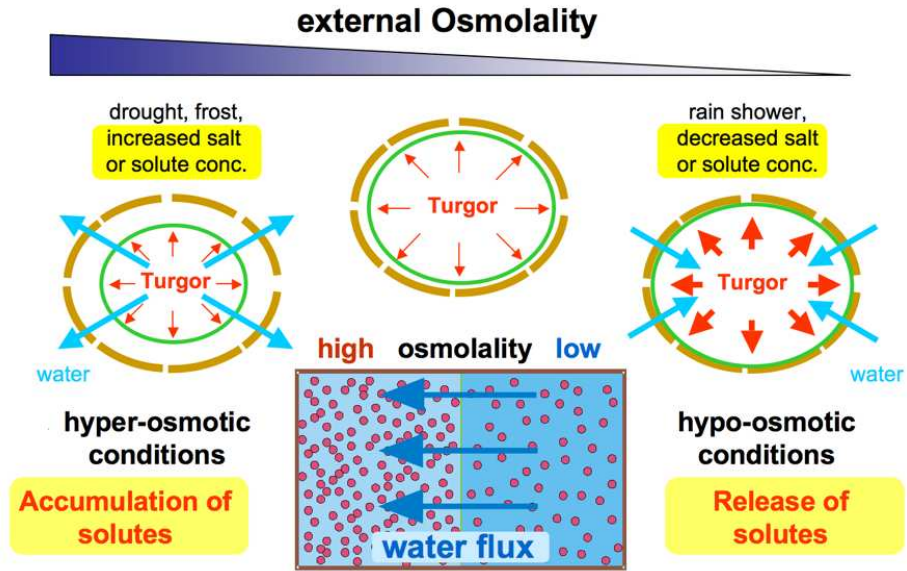
1321 [1] Peter *et al.*, (1996) *J. Bacteriol.* **178**: 5229-5234; [2] Kappes, *et al.*, (1996) *J. Bacteriol.*
1322 **178**: 5071-5079; [3] Sleator *et al.*, (1999) *Appl Environ Microbiol* **65**: 2078-2083; [4] Lu *et*
1323 *al.*, (2004) *FEMS Microbiol Lett* **235**: 393-399; [5] Vermeulen *et al.*, (2004) *Extremophiles* **8**:
1324 175-184; [6] Baliarda *et al.*, (2003) *Curr Microbiol* **47**: 347-351; [7] Naughton *et al.*, (2009)
1325 *Appl Environ Microbiol* **75**: 2802-10; [8] Fan *et al.*, (2003) *Mol Microbiol* **50**: 537-48; [9]
1326 Laloknam *et al.*, (2006) *Appl Environ Microbiol* **72**: 6018-26; [10] Ly *et al.*, (2004) *J*
1327 *Bacteriol* **186**: 296-306; [11] Boscari *et al.*, (2002) *J Bacteriol* **184**: 2654-2663; [12] Todd *et*
1328 *al.*, (2010) *Environ Microbiol* **21**: 327-343; [13] Steger *et al.*, (2004) *FEBS Lett* **573**: 155-
1329 160; [14] Kuhlmann *et al.*, unpublished data; [15] Weinand *et al.* (2007) *Appl Microbiol*
1330 *Biotechnol* **76**: 701-708; [16] Chen and Beattie, (2008) *J Bacteriol* **190**: 2717-2725; [17]
1331 Lamark *et al.*, (1991) *Mol. Microbiol* **5**: 1049-1064; [18] Rosenstein *et al.*, (1999) *J Bacteriol*
1332 **181**: 2273-2278; [19] Eichler *et al.*, (1994) *Mol Microbiol* **13**: 775-786; [20] Schulze *et al.*,
1333 (2010) *Nature* (in press)

1334 * The BetT protein from *Haemophilus influenzae* was suggested to function in the uptake of
1335 [Fan *et al.*, (2003) *Mol Microbiol* **50**: 537-48] but the actual substrate has not been
1336 unambiguously determined.

1337

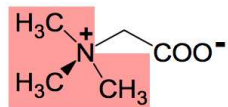
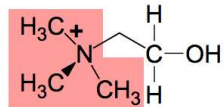
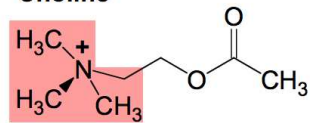
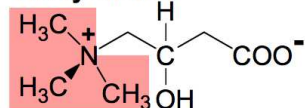
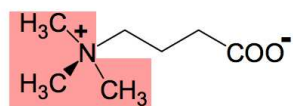
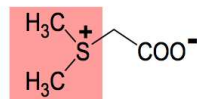
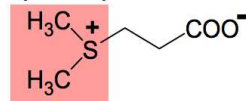
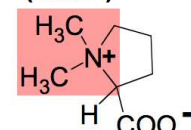
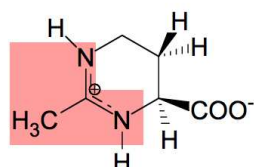
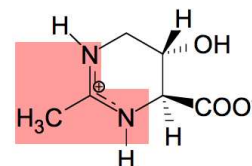
1338

1339



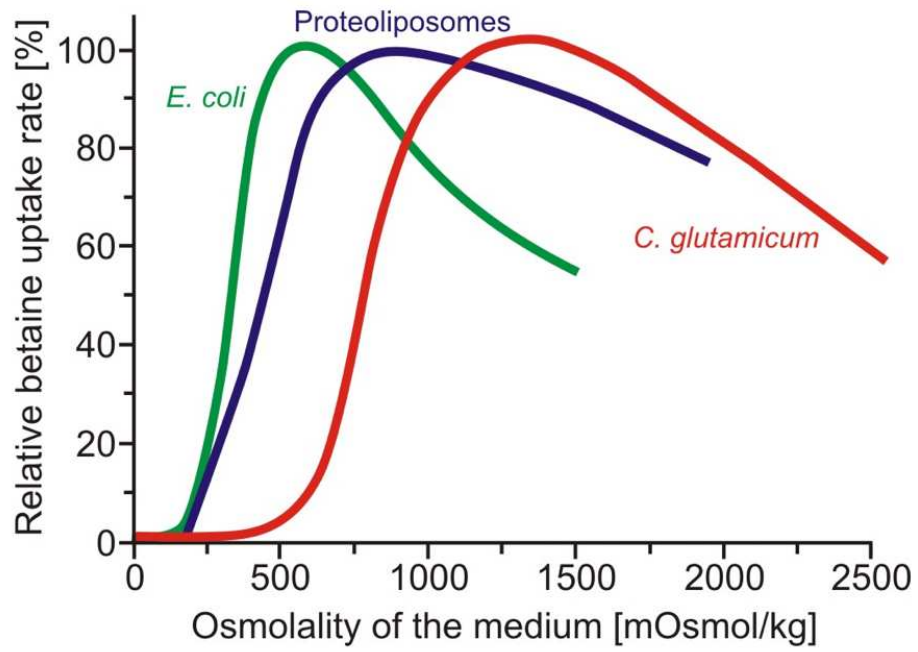
339x262mm (72 x 72 DPI)

review

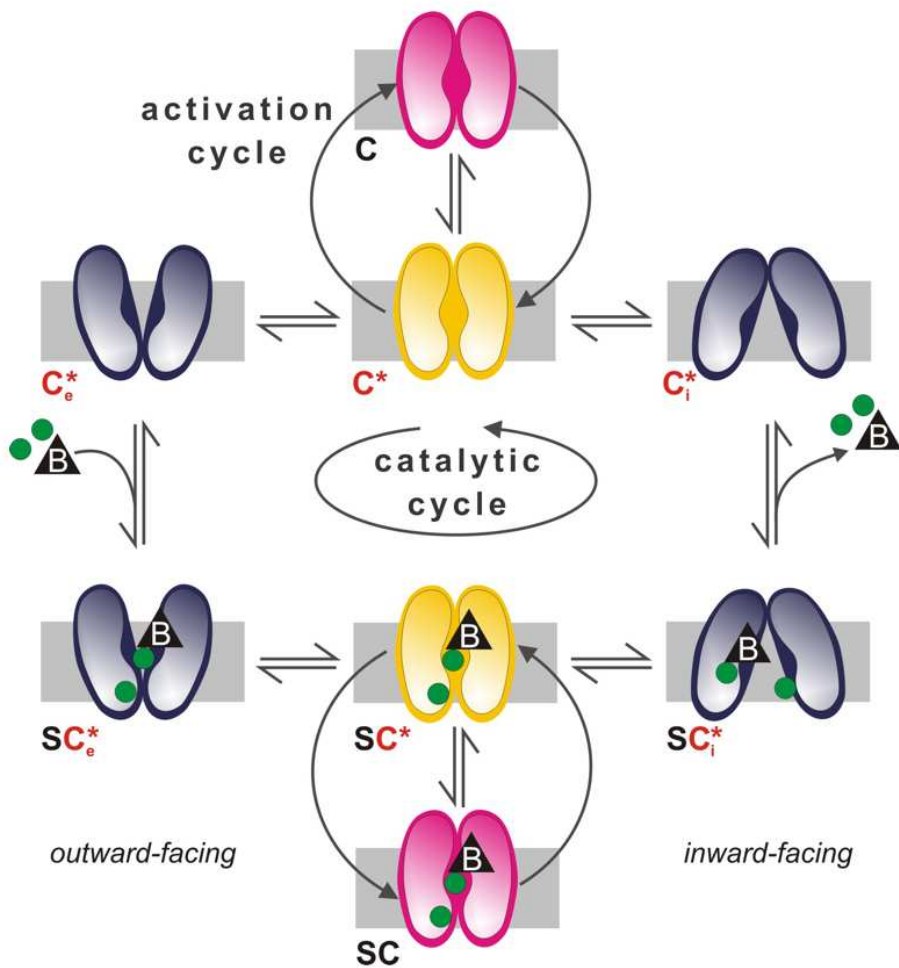
**Glycine betaine****Choline****Acetylcholine****L-carnitine****γ-Butyrobetaine****Dimethylsulfonioacetate (DMSA)****Dimethylsulfoniopropionate (DMSP)****Prolinebetaine****Ectoine****Hydroxyectoine**

467x347mm (72 x 72 DPI)

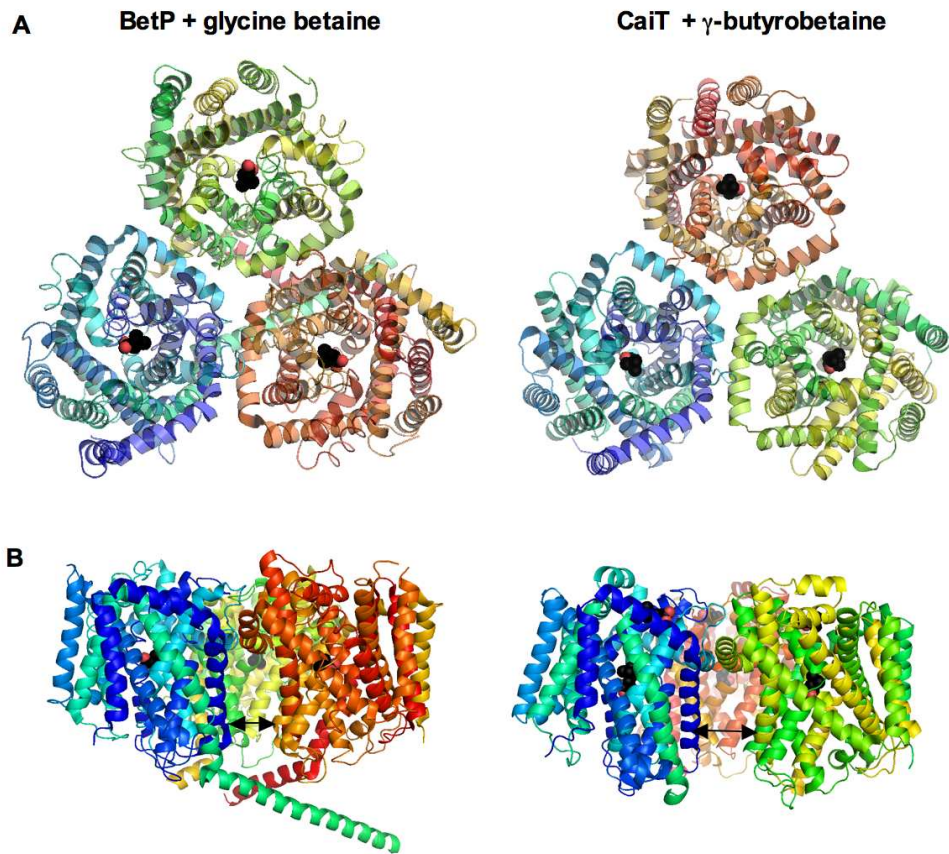
review



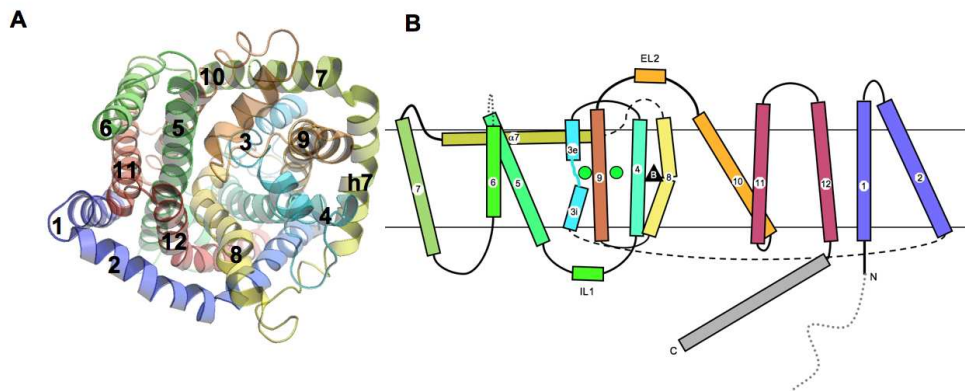
339x281mm (72 x 72 DPI)



304x317mm (72 x 72 DPI)

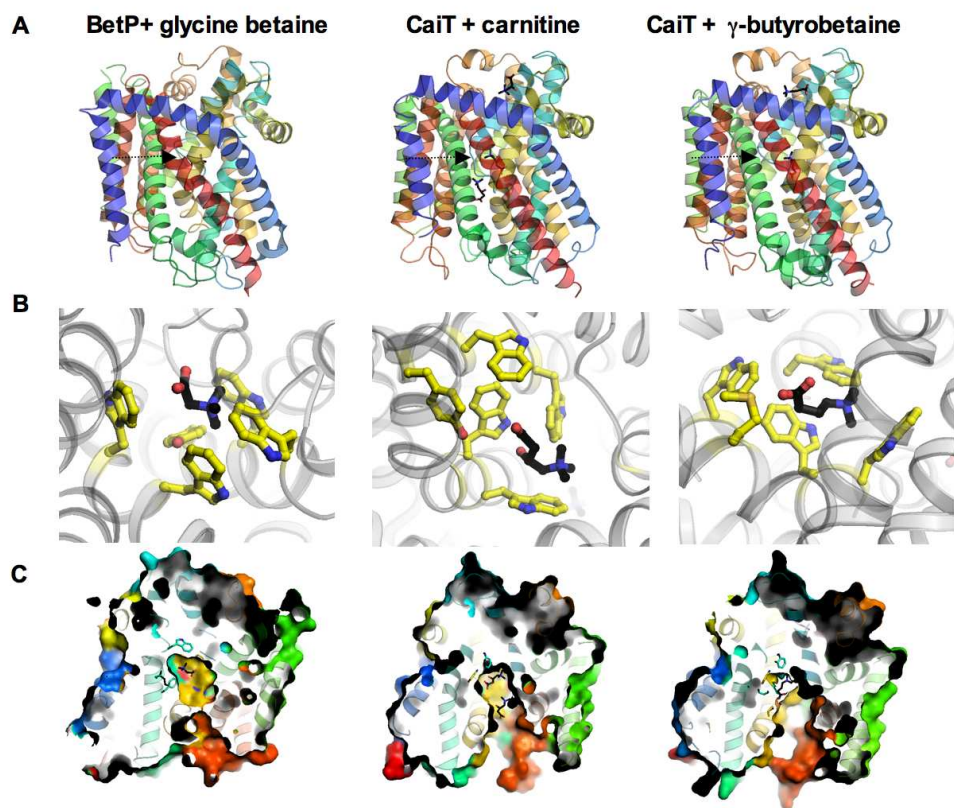


420x366mm (72 x 72 DPI)



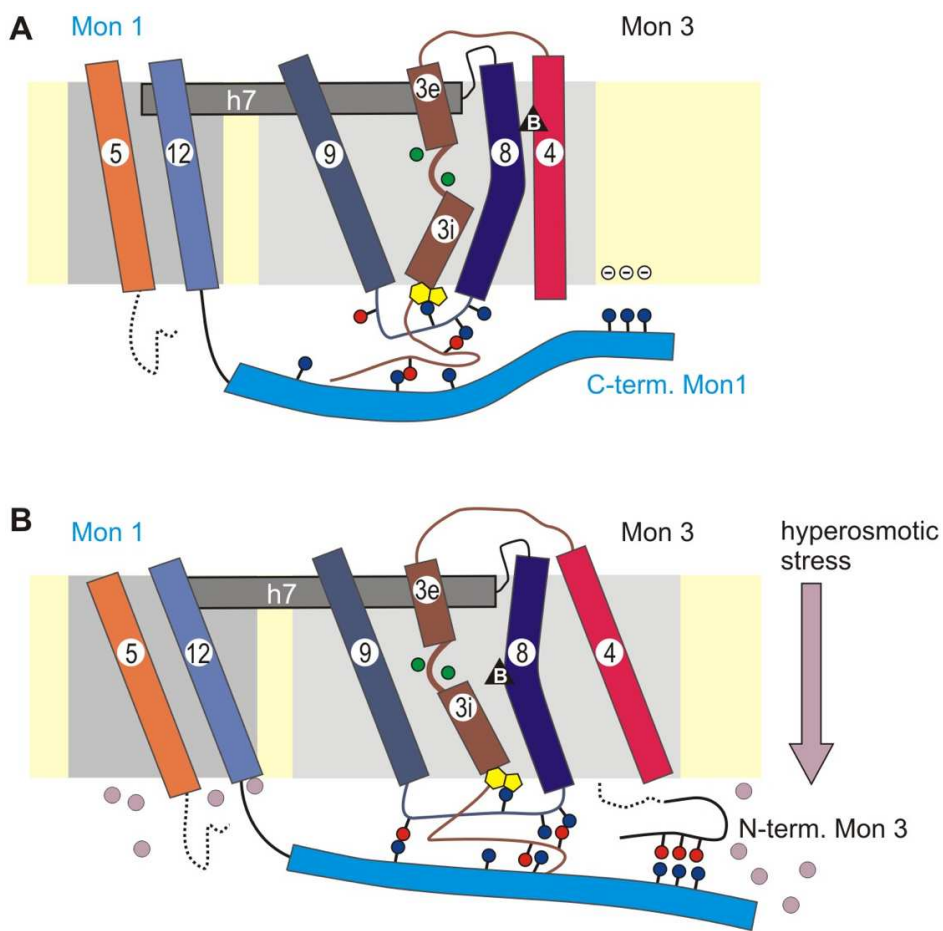
454x212mm (72 x 72 DPI)

Peer Review



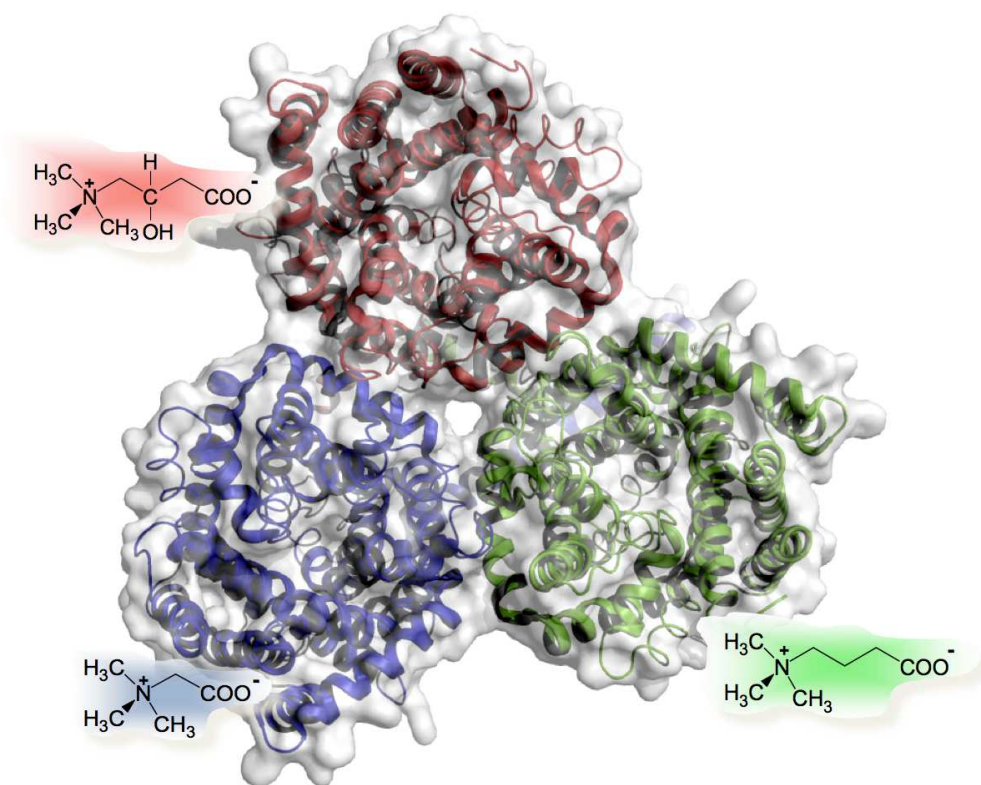
443x375mm (72 x 72 DPI)

iew



422x392mm (72 x 72 DPI)





423x330mm (72 x 72 DPI)

view

Caption for the proposed cover picture:

Top-down view on the trimer of the BetP BCCT-carrier and substrates for BetP (glycine betaine; blue) and the L-carnitine: γ -butyrobetaine (red and green, respectively) antiporter CaiT, a member of the BCCT-family.

For Peer Review