Membrane topology and mutational analysis of the osmotically activated BetT choline transporter of *Escherichia coli*

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For osmoprotection, *Escherichia coli* can synthesize glycine betaine from externally supplied choline by the Bet system (*betTIBA* products). The major carrier of choline is the high-affinity, proton-driven, secondary transporter BetT, which belongs to the BCCT family of transporters. Fusion proteins consisting of N-terminal fragments of BetT linked to β -galactosidase (LacZ) or alkaline phosphatase (PhoA) were constructed. By analysis of 51 fusion proteins with 37 unique fusion-points, the predictions that BetT comprised 12 membrane-spanning regions and that its N- and C-terminal extensions of about 12 and 180 amino acid residues, respectively, were situated in the cytoplasm were confirmed. This is believed to represent the first experimental examination of the membrane topology of a BCCT family protein. Osmotic upshock experiments were performed with spectinomycin-treated *E. coli* cells that had expressed the wild-type or a mutant BetT protein during growth at low osmolality (160 mosmol kg⁻¹). The choline transport activity of wild-type BetT increased tenfold when the cells were stressed with 0.4 M NaCl (total osmolality 780 mosmol kg⁻¹). The peak activity was recorded 5 min after the upshock and higher or lower concentrations of NaCl reduced the activity. Deletions of 1–12 C-terminal residues of BetT caused a gradual reduction in the degree of osmotic activation from ten- to twofold. Mutant proteins with deletion of 18–101 residues displayed a background transport activity, but they could not be osmotically activated. The data showed that the cytoplasmic C-terminal domain of BetT plays an important role in the regulation of BetT activity and that C-terminal truncations can cause BetT to be permanently locked in a low-transport-activity mode.

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INTRODUCTION

Escherichia coli adapts to environments of elevated osmotic strength by accumulation of potassium glutamate together with compatible solutes. The organism has two well-characterized transporters, ProU and ProP, for uptake of the compatible solutes glycine betaine, proline and ectoine. ProU is an ATP-binding cassette (ABC) transporter (May *et al.*, 1986), whereas ProP is a secondary transporter driven by the proton-motive force. ProP belongs to the major facilitator superfamily and possesses a large cytoplasmic C-terminal domain (Culham *et al.*, 2000; Zoetewey *et al.*, 2003). These transporters are osmotically activated at both the transcriptional and post-translational levels. *E. coli* also has two metabolic pathways for the synthesis of compatible solutes. These are the osmoregulated Ots system for UDP-glucose-dependent synthesis of trehalose (Giæver *et al.*, 1988) and the Bet system for synthesis of glycine betaine from externally supplied choline (Landfald & Strøm, 1986).

The *bet* regulon comprises three structural genes: *betT* encoding a choline transporter, *betA* encoding choline dehydrogenase and *betB* encoding betaine aldehyde dehydrogenase. In addition, *betI* encodes a DNA-binding and choline-sensing regulator. The *betIBA* and the *betT* genes are divergently organized and the promoters of *betI* and *betT* overlap (Lamark *et al.*, 1991, 1996; Røkenes *et al.*, 1996).

BetT is a proton-driven secondary transporter (Lamark *et al.*, 1991) belonging to the BCCT family which comprises transporters for quaternary ammonium compounds. These transporters are predicted to have 12 membrane-spanning regions and to have their hydrophilic N- and C-termini located in the cytoplasm (Saier *et al.*, 1999). However, the membrane topology has not been tested experimentally for any such transporter. In this investigation, we have therefore probed and verified this domain structure of BetT by constructing a series of fusion proteins with C-terminally linked alkaline phosphatase (PhoA) or β -galactosidase (LacZ). This analysis is based on the well-established fact that the PhoA moiety of hybrid proteins displays phosphatase activity only when translocated to the periplasmic space

Abbreviation: ABC, ATP-binding cassette; XP, 5-bromo-4-chloro-3-indolyl phosphate.

(Manoil & Beckwith, 1986), whereas the LacZ moiety must remain in the cytoplasm to be enzymically active (Froshauer *et al.*, 1988).

The homologous BetT transporters for choline of *E. coli* (Andresen *et al.*, 1988) and *Haemophilus influenzae* (Fan *et al.*, 2003) are activated at the level of transport by osmotic stress, despite the fact that choline is not a compatible solute per se (Styrvold *et al.*, 1986). These transporters seem to have much the same properties as glycine betaine transporters of the BCCT family; e.g. BetP of *Corynebacterium glutamicum* (Farwick *et al.*, 1995), BetS of *Sinorhizobium meliloti* (Boscari *et al.*, 2002) and OpuD of *Bacillus subtilis* (Kappes *et al.*, 1996). However, there are intriguing differences between these transporters that do not reflect their substrate specificity. BetS (Boscari *et al.*, 2002) and the two BetT transporters (Lamark *et al.*, 1991; Fan *et al.*, 2003; present study) have a large deduced C-terminal domain comprising about 170–180 amino acid residues, whereas this domain of OpuD (Kappes *et al.*, 1996) and BetP (Peter *et al.*, 1996) comprises only about 30 and 60 residues, respectively. In addition, BetS and BetP have an N-terminal hydrophilic domain of about 50–60 residues, which is much larger than the ones found in the three other transporters.

ProP of *E. coli* (Racher *et al.*, 1999), BetP of *C. glutamicum* (Rübenhagen *et al.*, 2000) and the ABC transporter for glycine betaine, OpuA, of *Lactococcus lactis* (van der Heide & Poolman, 2000) have been purified and reconstituted into liposomes and shown to work as both osmosensors and osmoregulators independently of other proteins. Although these transporters are widely different, they all respond to the osmotic conditions in the cytoplasm and the lumen of liposomes. Only ionic osmolytes (particularly K^+) activate OpuA and BetP (van der Heide *et al.*, 2001; Rübenhagen *et al.*, 2001), whereas ProP also responds to organic molecules in a size-dependent manner (Culham *et al.*, 2003). BetP is a trimer in the cytoplasmic membrane and each monomer is predicted to have a transport pathway (Ziegler *et al.*, 2004). ProP exists in monomer–dimer equilibrium *in vivo* and dimerization may be mediated by the formation of a C-terminal antiparallel coiled-coil structure which stabilizes ProP in an active conformation (Hillar *et al.*, 2005).

The osmotic activation of *E. coli* BetT has not previously been studied in detail. In this investigation, we have characterized the *in vivo* transport activity of wild-type BetT and BetT mutant proteins carrying deletions or amino acid substitutions in the C-terminal hydrophilic domain. Whereas C-terminal deletions of BetP of *C. glutamicum* led to permanent activation of this glycine betaine transporter (Peter *et al.*, 1998; Morbach & Krämer, 2003), deletions of 18–101 amino acid residues of BetT locked it in a low-activity state of choline transport.

METHODS

Bacterial strains, plasmids and phage. These are listed in Tables 1, 3 and 4.

Growth media. The rich medium used was LB. The minimal media used were medium 63 (M63) with 0.4% (v/w) glucose and half-strength M63 (Miller, 1972). The latter medium contained half the amount of the inorganic salts and the full amount of glucose. The osmotic strength of the media was increased by addition of NaCl or sucrose as stated in the text. LB agar containing the indicator dye 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 $\mu\text{g ml}^{-1}$) was used for selection of strains with alkaline phosphatase activity (Manoil, 1991). When required, ampicillin (Amp; 100 $\mu\text{g ml}^{-1}$), chloramphenicol (Chl; 30 $\mu\text{g ml}^{-1}$) or tetracycline (Tet; 15 $\mu\text{g ml}^{-1}$) was added to the media.

Standard molecular biology techniques and strain constructions. Restriction endonuclease digestion, ligation and agarose gel electrophoresis of DNA were performed in accordance with standard protocols (Sambrook *et al.*, 1989). Plasmid DNA was prepared by the midi-kit from Qiagen or the Wizard miniprep kit from Promega. DNA was extracted from agarose gels using the Qiaex or the Qiaquick kit from Qiagen. DNA sequencing was performed using cycle sequencing with the AmpliTaq kit from PE Applied Biosystems. PCR was performed utilizing the Expand High Fidelity PCR System from Roche or the *PfuTurbo* DNA polymerase from Stratagene. DH5 α was used as a standard transformation host and transformation was performed by the method of Chung *et al.* (1989) or by electroporation as described by Hanahan *et al.* (1991). By selecting for Tet^R and scoring for LacZ⁺ phenotype, $\Delta(\text{argF-lac})\text{U169}$ and *zah::Tn10* of SH205 was cotransduced by P1 into strain JP8442 by the method described by Miller (1972). The Tet^R marker was deleted from the resulting transductant (AT100) by using the method described by Bochner *et al.* (1980), yielding strain AT101. Strain AT300 was constructed by deleting the Tet^R marker in MHK-1 by using the same method (Table 1). The $\Delta(\text{argF-lac})\text{U169}$ deletion encompasses the *betTIBA* gene cluster.

Construction of *betT-phoA* fusion plasmids. The *Bsp*HI site of the low-copy-number vector pACYC184 was filled in using Klenow polymerase, and the *phoA* gene from pUI310 was then ligated as a 1.5 kb *Hind*III–*Bam*HI fragment into the same sites of the vector. The *betT* gene from pFF221 was then ligated as a 2.7 kb *Pvu*II–*Xmn*I fragment into the *Hind*III site, which was first made blunt by Klenow polymerase. The resulting plasmid pAT3 carried *betT* including its stop codon upstream of a signal-less and unexpressed *phoA* reporter gene, separated by a linker containing *Bsp*HI, *Pst*I, *Xba*I and *Bam*HI restriction sites in that order. These sites together with the *Xba*I and *Pvu*II sites upstream of *betT*, the *Sfi*I, *Bsi*WI and *Nde*I sites within *betT* and a *Kpn*I site downstream of *phoA* were used for construction of plasmids with in-frame *betT-phoA* fusions, using methods 1 to 3 described below. The constructed plasmids can be identified by their fusion-point numbers listed in Table 3.

1. *Random deletions.* pAT3 was linearized with *Bsp*HI and *Pst*I to create a 5' overhang downstream of *betT* and a 3' overhang protected from exonuclease activity upstream of *phoA*. Time-dependent exonuclease III (Erase-a-base kit, Promega) treatment was then applied to introduce 3' deletions in *betT*. The resulting pAT3 fragments were then blunted with S1 nuclease and Klenow DNA polymerase before recircularization with T4 DNA ligase and transformation into strain AT101. Plasmids from transformants forming blue colonies on XP indicator plates were analysed by restriction digestion and DNA sequencing, thereby identifying fusion-point numbers 2, 4–10, 12–17, 19–24, 26 and 28–32 (Table 3).

2. *PCR based constructions.* To create fusion-point number 37, a PCR fragment was amplified from pAT3 using primer pair 1 (Table 2). One primer was homologous to a region upstream of the *Nde*I site in *betT* and the second introduced a *Bam*HI site over the last coding triplet and also a silent mutation (GAC→GAT) in the Asp-676 codon of *betT*. PCR-amplified DNA was cloned as a *Nde*I–*Bam*HI fragment into the

Table 1. *E. coli* strains, bacteriophage and plasmids used in the construction procedures

Strain, plasmid or bacteriophage	Genotype or relevant characteristics*	Reference or source†
Strains		
DH5 α	ϕ 80d <i>lacZ</i> Δ M15 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 λ^- relA1 Δ(argF-lac)U169</i>	BRL
JP8442	W3110 (Δ <i>phoA8 tsx</i>)	A. J. Pittard
SH205	HfrC <i>phoA8 glpD3 glpR2 relA1 tonA22 (λ)Δ(argF-lac)U169 <i>zah-735::Tn10; Tet^R</i></i>	Schweizer & Boos (1983)
AT100	JP8442 Δ (argF-lac)U169 <i>zah-735::Tn10; Tet^R</i>	This work
AT101	AT100; Tet ^S	This work
MHK-1‡	MC4100 Δ (<i>putPA</i>)101 Δ (<i>proU</i>)600 Δ (<i>proP</i>)3::Tn10 Δ (argF-lac)U169; Tet ^R	E. Bremer
AT300	MHK-1; Tet ^S	This work
Plasmids		
pACYC184	Low-copy-number cloning vector; Chl ^R Tet ^R	NEB
pGEM7-Zf(+)	High-copy-number cloning vector; Amp ^R	Promega
pUI310	pUC19 derivative carrying <i>phoA</i> ; Amp ^R	Varga & Kaplan (1989)
pXCA601	<i>lacZ</i> vector; Tet ^R	Adams <i>et al.</i> (1989)
pFF221	pBR328 derivative carrying <i>betTIBA</i> ; Amp ^R Chl ^R	Andresen <i>et al.</i> (1988)
pAT3	Derivative of pACYC184 carrying <i>betT</i> upstream of a signal-less and unexpressed <i>phoA</i> reporter gene; Chl ^R	This work
pAT5	' <i>lacZ</i> and transcriptional terminator from pXCA601 was ligated as a 3.5 kb <i>Bam</i> HI– <i>Hind</i> III fragment into the same sites in pGEM7-Zf(+); Amp ^R	This work
pAT6	<i>betT</i> from pAT3 was ligated as a 2.9 kb <i>Xba</i> I fragment into pGEM7-Zf(+); Amp ^R	This work
Phage		
P1	<i>cml crl-100</i>	Miller (1972)

*Amp^R, ampicillin resistance; Chl^R, chloramphenicol resistance; Tet^R, tetracycline resistance.

†A. J. Pittard, University of Melbourne, Australia; BRL, Bethesda Research Laboratories; E. Bremer, Philipps University Marburg, Germany; NEB, New England Biolabs.

‡The Δ (argF-lac)U169 deletion encompasses the *betTIBA* gene cluster.

same sites of pAT3, thereby replacing the original *betT* fragment. For generation of fusion-point numbers 1, 3, 11, 18 and 27, *Bam*HI sites were introduced at other locations in *betT* by using primer pairs 2 to 6. The first oligo in each pair was identical and could bind upstream of the *Pvu*I site preceding *betT*. The PCR-generated fragments were digested with *Pvu*I and *Bam*HI and cloned into the same sites of pAT3. Fusion-point number 36 was constructed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). A *Bam*HI site was introduced just downstream of *betT* codon number 653 in pAT6 using primer pair 7. A *Bsi*WI–*Bam*HI fragment of PCR-amplified DNA was then cloned into the same sites of pAT3. The PCR amplified parts of these fusion plasmids were sequenced.

3. *Use of restriction sites.* pAT3 was linearized by digestion with the restriction enzymes *Sfi*I–*Pst*I, *Bsi*WI–*Bam*HI or *Nde*I–*Pst*I. The pAT3 fragments were treated with T4- or Klenow DNA polymerase before religation, thereby creating fusion-point numbers 25, 33 and 35. The junctions of these plasmids were sequenced. Fusion plasmids generated by methods 2 and 3 were finally transformed into strain AT101.

Construction of *betT-lacZ* fusion plasmids. A *lacZ* gene derivative without the eight first codons was excised as a 3.5 kb *Bam*HI–*Kpn*I fragment from pAT5 and ligated into the same sites of selected pAT3-derived plasmids, thereby replacing their in-frame *betT-phoA* with an in-frame *betT-lacZ* at fusion-points 1, 3, 11, 12, 18, 20, 22,

23, 27, 31, 32 and 35–37 (Table 3). To create an in-frame *betT-lacZ* fusion at point number 34, we used the QuickChange strategy to introduce a *Bam*HI site just downstream of codon 576 of the *betT* gene by using primer pair 8 and pAT6. The resulting mutant *betT* fragment was cloned as a *Xba*I fragment into pAT3 linearized with the same enzyme. The resulting plasmid was then cut with *Bam*HI and *Kpn*I and joined with the *lacZ*-containing fragment from pAT5. The plasmids were finally transformed into strain AT101.

C-terminal deletions and amino acid substitutions in BetT.

The QuickChange mutagenesis strategy was applied to introduce stop codons at selected sites in *betT*. The procedures were performed with primer pairs 9–16 (Table 2) using pAT6 as template. The resulting plasmids encoded BetT proteins with C-terminal deletions of 1–101 residues. The mutant *betT* genes were first cloned as *Xba*I fragments into pAT3, and to limit the necessary DNA sequencing the *Bsi*WI–*Bam*HI fragments carrying the mutated 3' ends of *betT* were again subcloned into pAT3. Mutant BetT proteins with C-terminal amino acid substitutions were made by the same strategy using the primer pairs 17–19. The plasmids were finally transformed into strain AT300. The plasmids constructed and the characteristics of the BetT mutant proteins are listed in Table 4.

Enzyme assays. Alkaline phosphatase activity of cells was assayed by measuring the rate of *p*-nitro phenyl phosphate hydrolysis as

Table 2. Primers used for construction of BetT fusions and BetT mutants with deletions or substitutions

Primer pair	Fusion-point no. or BetT construct*	Sequence (5'→3')†
1	37	GTCATCTGGATTTGTTGG AATCaAGgaTCCGGGAACATCA
2	1	CCCTGAAATAGTGGCTGATG (Fwd2) CACCACCGGATccATTTTGT
3	3	Fwd2 CAGGCTGggaTCCGGTTTG
4	11	Fwd2 CCACTGAGgGAtCcATCGG
5	18	Fwd2 CGCGACATTAgGaTCCGATA
6	27	Fwd2 TCTTTAgGatCCGAGGTGAA
7	36	CCGATATTCTTGACCAGgAtccGCGGCACCTTAAC GTTAAGGTGCCGcggaTcCTGGTCAAGAATATCGG
8	34	AAGCCTGCCACCGGAtctGGACAGCAGTTGGGTC GACCCAACTGCTGTCCaggaTCCGGTGGCAGGCTT
9	BetT676	CGGACtaGTGATTGTTATTG CAATAACAATCACtaGTCCG
10	BetT675	TGTTCCCGtAaGCGTGATTG CAATCACGCtTaCGGGAACA
11	BetT671	GTGAAGCGCCGGCCATAGCtaGATGTTCC GGAACATCtaGCTATGGCCCGGCGCTTCAC
12	BetT665	CATCTCCATCGTtAAGCGCCGGGCCATAGC GCTATGGCCCGGCGCTTaACGATGGAGATG
13	BetT659	GCGGCACCTTAACTgaATTTCATCTCCATCG CGATGGAGATGAATtcAGTTAAGGTGCCGC
14	BetT653	CGATATTCTTGACCAGTAgGAGCGGCACC GGTGCCGCTCcTACTGGTCAAGAATATCG
15	BetT590	GATTTGTTGGTGCATtaGGGCGAAGAG CTCTTCGCCctATGCACCAACAAATC
16	BetT576	GCCTGCCACCGtAAGAGGGACAGC GCTGTCCCTCTTaCGGTGGCAGGC
17	BetT677-V672A-M673A	CCATAGCGcGgcGTTCCCGGACGCGTGATT AATCACGCGTCCGGGAACgcCgCGCTATGG
18	BetT677-M673A-F674A	GCCATAGCGTgGcGgcCCCGGACGCGTGATTG CAATCACGCGTCCGGGgcCgcCACGCTATGGC
19	BetT677-F674A-P675A-D676G	CCGGGCCATAGCGTGATGgcCgCaGcCGCGTGATTG CAATCACGCGgCtGcGgcCATCACGCTATGGCCCGG

*As referred to in Tables 3 and 4.

†The forward primer in pairs 2–6 is identical (Fwd2). Restriction sites, stop codons and changed triplets are underlined. Nucleotides differing from wild-type *betT* are indicated by lower-case letters.

described by Manoil (1991). The cells were grown at 37 °C in LB to an OD₆₀₀ of 0.5–1.0. To prevent the slow activation of cytoplasmic forms of alkaline phosphatase in non-growing cells, iodoacetamide was added to the assay mixture at a final concentration of 1 mM (Derman & Beckwith, 1995). β-Galactosidase activity was assayed by measuring the rate of hydrolysis of *o*-nitrophenyl β-D-galactoside as described by Miller (1972). The cells were grown at 37 °C in LB or half-strength M63 to an OD₆₀₀ of 0.5–1.0 and then permeabilized by treatment with SDS and chloroform. Units of enzyme activity were calculated according to the references cited above. The values given

in Table 3 are the means of duplicate determinations from at least three independent cultures and standard deviation from the mean was in general less than 20 %.

BetT–PhoA fusion protein levels. To determine the expression level of the fusion proteins, whole cells were subjected to SDS-PAGE and Western blotting. The cells were grown in M63 to an OD₆₀₀ of 0.5–1.0, harvested and resuspended in 50 mM Tris pH 8.0 to an OD₆₀₀ of 20. Then 20 µl samples of this suspension were separated by SDS-7.5 % PAGE and proteins were transferred (1 h at 80 V) to

Table 3. Description and enzyme activities of BetT–PhoA and BetT–LacZ fusion proteins

Fusion-point no.	Last BetT residue	Alkaline phosphatase activity (Rel. U)*	β -Galactosidase activity (Rel. U)†
1	Lys-12	0	910 (7400)
2	Gly 44	100	–‡
3	Pro-89	0	160 (1700)
4	Gly 106	110	–
5	Met-110	100	–
6	Gln-130	130	–
7	Ala-135	90	–
8	Gln-137	120	–
9	Ala-138	130	–
10	Trp-141	100	–
11	Pro-189	0	150 (1200)
12	Ala-207	60	50 (670)
13	Leu-210	100	–
14	Gly 211	140	–
15	Ser-230	140	–
16	Ala-232	160	–
17	Ala-235	160	–
18	Ser-261	0	170 (1400)
19	Leu-273	120	–
20	Leu-276	170	0 (0)
21	Gly 301	180	–
22	Ala-339	1	40 (430)
23	Ser-367	180	0 (0)
24	His-379	130	–
25	Tyr-397	160	–
26	Gln-402	180	–
27	Ser-437	0	110 (1500)
28	Met-467	170	–
29	Ala-473	220	–
30	Asn-476	210	–
31	Leu-483	190	0
32	Ala-494	1	60 (660)
33	Tyr-568	0	–
34	Pro-576	–	300 (2800)
35	His-590	0	280 (2600)
36	Gln-653	0	270 (1900)
37	Asp-676	0	270 (2400)

*The relative units (Rel. U) of phosphatase activity were calculated as described by Manoil (1991). The cells were grown in LB.

†The relative units (Rel. U) of β -galactosidase activity were calculated as described by Miller (1972). The cells were grown in LB or half-strength M63 (numbers in parentheses).

‡–, Constructs not made.

a nitrocellulose membrane (Millipore). The membrane was blocked with 1% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 150 mM NaCl) before detection of BetT–PhoA fusion proteins with rabbit anti-*E. coli* alkaline phosphatase (Polysciences) as the primary antibody. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) was used as the secondary antibody and BCIP/NBT Blue (Sigma) was used as the liquid substrate. The BetT–PhoA fusion proteins tested are stated in the text.

Transport measurements. Strain AT300 carrying the relevant plasmids was grown at 37 °C in half-strength M63 or in M63 supplemented with 0.3 M NaCl to an OD₆₀₀ of 0.4–0.6. Spectinomycin was added to a final concentration of 1 mg ml^{–1} to prevent further protein synthesis, and the cultures were maintained at room temperature for no longer than 2 h prior to the transport assay. The choline transport capacity of the cells was activated by addition of NaCl or sucrose at 37 °C; the solute concentrations (up to 0.8 M)

Table 4. Osmotic activation of choline uptake in *E. coli* expressing wild-type BetT or BetT mutants with C-terminal deletions or amino acid substitutions

Plasmid	Description of BetT protein expressed	Initial rate of choline uptake [nmol min ⁻¹ (mg protein) ⁻¹]* when tested in†:		Choline accumulated at steady-state level [nmol (mg protein) ⁻¹ ‡ when tested in§:	
		Half-strength M63	Half-strength M63 + 0.4 M NaCl	Half-strength M63	Half-strength M63 + 0.4 M NaCl
pAT3	BetT677 (wild-type)	19 ± 3	181 ± 11	36	360
pAT101	BetT676 (Δ1)	16 ± 6	162 ± 19	23	310
pAT102	BetT675 (Δ2)	15 ± 5	162 ± 19	23	340
pAT103	BetT671 (Δ6)	16 ± 3	88 ± 15	31	160
pAT104	BetT665 (Δ12)	15 ± 3	36 ± 5	18	52
pAT105	BetT659 (Δ18)	15 ± 6	12 ± 3	17	23
pAT106	BetT653 (Δ24)	14 ± 5	12 ± 4	14	17
pAT107	BetT590 (Δ87)	14 ± 6	14 ± 4	12	12
pAT108	BetT576 (Δ101)	9 ± 3	14 ± 6	12	16
pAT109	BetT677-V672A-M673A	19 ± 8	128 ± 10	23	210
pAT110	BetT677-M673A-F674A	19 ± 5	67 ± 6	20	93
pAT111	BetT677-F674A-P675A-D676G	12 ± 2	78 ± 11	20	65

*The values given are the means ± SD of at least ten determinations from two to four separate cell cultures.
†Strain AT300 carrying the BetT expressing plasmids was grown in half-strength M63 and tested for initial uptake rate under the same conditions, and when 0.4 M NaCl was added 5 min prior to the addition of choline.
‡The standard deviations from the mean of the values given were within ± 15 %.
§Steady-state levels of choline accumulation were based on at least five samples taken after incubation with substrate for at least 30 min.

and time periods (up to 20 min) used are stated in the text. The uptake reaction was started by adding [¹⁴C]choline to the culture. The choline concentration routinely used was 200 μM; the specific activity was 150 nCi μmol⁻¹ (5.6 kBq μmol⁻¹). In the experiments for measuring kinetic parameters, the specific activity of the substrates varied from 150 to 1500 nCi μmol⁻¹ (5.6–56 kBq μmol⁻¹) depending on the substrate concentration, which was varied from 2.5 to 50 μM. After time intervals as stated in the text, the reaction was stopped by withdrawal and filtration of 0.5 or 1.0 ml samples. The filters (pore size 0.45 μm) were immediately washed once with 10 ml M63 containing 0.3 M NaCl and 0.5 M sucrose. The filters were then solubilized in 5 ml Filter-Count LSC cocktail (Packard) and the radioactivity was determined by liquid scintillation counting.

Other methods. Cell protein was determined by the Lowry method after treating the cells with 0.1 M NaOH at 95 °C for 10 min. Bovine serum albumin (fraction V, Sigma) was used as standard. The osmolality of growth media was measured as mosmol kg⁻¹ by freezing-point depression.

RESULTS

Predicted membrane topology of BetT

Prediction of the topology (Fig. 1) of the BetT transporter (677 amino acid residues) was based on the TMHMM method, which takes into account the expected hydrophobicity of transmembrane regions and the abundance of positively charged residues on the cytoplasmic side of the membrane (Krogh *et al.*, 2001). Other databases (e.g. National Center for Biotechnology Information) identify

the same 12 transmembrane regions in BetT, but with slightly different boundaries.

Investigation of the membrane topology by the use of PhoA and LacZ fusion proteins

The low-copy-number plasmid pAT3 carried *betT* with its native promoter upstream of an unexpressed *phoA* reporter gene which in a single cloning step could be replaced with a *lacZ* reporter gene in the same reading frame. The *betIBA* operon was not present, except for a 201 bp region of *betI*. Random *betT*–*phoA* fusions were made by joining the gene derivatives after unidirectional exonuclease digestion of the 3' end of *betT* (see Methods). Transformants of strain AT101 displaying alkaline phosphatase activity were selected on indicator plates with the chromogenic substrate XP. The host organism AT101 carried Δ*phoA*8 and Δ(*argF*–*lac*)U169, which encompasses the *betTIBA* gene cluster. By analysis of plasmids from 24 dark blue and two light blue colonies, we identified 26 different in-frame fusion-points (Table 3, Fig. 1). Ten of them (numbers 2, 6–10, 15, 21, 24 and 30) were located in each of the six predicted periplasmic loops of BetT. Four (numbers 16, 17, 26 and 31) were located close to the periplasmic surface of putative ‘incoming’ transmembrane regions (N-termini pointing outwards). Thus PhoA was exported by the preceding transmembrane region. Ten (numbers 4, 5, 12–14, 19, 20, 23, 28 and 29) were located in putative ‘outgoing’ transmembrane regions and were preceded by at least nine native apolar residues. This

Fig. 1. Topological model of BetT. Membrane-spanning regions are shown as boxes numbered I to XII. The predicted BetT residues at the membrane boundaries are indicated with small numbers outside the boxes. P1 to P6 and C1 to C7 refer to the hydrophilic periplasmic and cytoplasmic segments, respectively. Circles numbered 1–37 indicate the point of fusion for BetT–PhoA and BetT–LacZ hybrid proteins as listed in Table 3.

number of apolar residues was previously reported to be sufficient for exporting the PhoA moiety of hybrid proteins to the periplasmic space (Calamia & Manoil, 1990).

When grown in LB, 23 of the transformants selected as dark blue colonies displayed levels of alkaline phosphatase activity ranging from about 90 to above 200 U (Table 3). The general tendency was that hybrid proteins with adjacent fusion-points displayed similar levels of activity and that their activity increased with the remaining length of BetT. In control experiments, we found that replacement of PhoA with LacZ at three of the fusion-points predicted to be in membrane-spanning regions (numbers 20, 23 and 31) gave hybrid proteins which did not confer β -galactosidase activity in cells grown in LB or half-strength M63.

An exception among fusion plasmids isolated from the dark blue colonies was the fusion-point number 12 (in membrane-spanning region number V; Fig. 1), which conferred an intermediate level of enzyme activity when fused with either PhoA or LacZ. Furthermore, the two *betT-phoA* transformants selected as light blue colonies on the XP indicator plates produced essentially no alkaline phosphatase activity when grown in LB medium. Their BetT–PhoA fusion-points were predicted to be in the cytoplasm (number 22) or near the cytoplasmic surface (number 32). Accordingly, the corresponding BetT–LacZ hybrid proteins displayed appreciable β -galactosidase activities (Table 3). However, cells producing these two LacZ hybrid proteins displayed reduced growth, even in rich medium.

The expression levels of 17 selected BetT–PhoA fusion proteins (numbers 2, 4, 6, 7, 12, 14, 15, 17, 19, 20–22, 24, 26, 28, 29 and 32) were determined by Western blotting using a primary antibody against the PhoA moiety. The fusion

proteins displayed increasing apparent molecular mass according to the expectation. They were present in comparable amounts in all cases examined except for fusion numbers 22 and 32, which gave very faint signals on the membrane (data not shown). These two fusion proteins were presumably subjected to proteolytic degradation since PhoA can not be folded correctly in the reducing conditions of the cytoplasm.

We used alternative *in vitro* techniques (see Methods) to generate five fusion-points in the large hydrophilic C-terminal end of BetT (numbers 33–37) and one fusion-point in each of the other predicted cytoplasmic regions that were not probed by the first series of hybrid proteins (numbers 1, 3, 11, 18 and 27). All BetT–PhoA hybrid proteins of this series were enzymically inactive when expressed in strain AT101, whereas the corresponding BetT–LacZ hybrids conferred a wide range of β -galactosidase activities to the host cells. The LacZ fusion with Lys-12 (number 1) was particularly active. For the remainder, the longer ones with fusion-points in the predicted C-terminal domain of BetT displayed the highest β -galactosidase activities.

Osmotic activation of wild-type BetT

Transport activity of plasmid encoded wild-type and mutant BetT proteins were measured in strain AT300, which carried the (*argF-lac*)U169 deletion, encompassing the *betTIBA* gene cluster (Table 1). This strain also carried deletions or mutations in all transporters for proline and glycine betaine, i.e. the measured choline uptake originates from the plasmid expressed *betT* genes.

As shown in Fig. 2(a), wild-type BetT was sufficiently expressed in strain AT300(pAT3) cells grown in

Fig. 2. Time-course of choline accumulation in strain AT300(pAT3) expressing wild-type BetT. Shown are the accumulation during a 30 min period (a) and during the first 1 min (b). ○, Cells grown and assayed in half-strength M63; ●, cells grown in half-strength M63 and exposed to 0.4 M NaCl 5 min prior to the addition of choline; ■, cells grown and assayed in full-strength M63 supplied with 0.3 M NaCl. The dashed lines in (b) represent an extrapolation to time zero; see text. The curves shown are representative for each condition.

half-strength M63 (160 mosmol kg⁻¹) to investigate the osmotic activation of the transporter; i.e. the uptake activity of spectinomycin-treated cells was greatly enhanced by addition of 0.4 M NaCl (total osmolality 780 mosmol kg⁻¹) 5 min before the transport assay started (see below). Furthermore, cells grown and tested in M63 with 0.3 M NaCl (720 mosmol kg⁻¹) displayed an even higher uptake activity, suggesting that the expression of the *betT* gene was stimulated by osmotic stress (Andresen *et al.*, 1988). It should be noted that the choline uptake rate decreased gradually with time and the choline content of the cells reached a maximum within 30 min. At this time, maximally 25% of the choline in the assay medium was taken up by the stressed cells; the start concentration was 200 µM.

As shown in Fig. 2(b), the gradual decrease in uptake rate was already noticeable during the first minute after the assay was started. Since the accumulation versus time curve was non-linear, we decided to define the initial uptake rate as the measured choline uptake after 30 s. The apparent choline uptake in spectinomycin-treated cells of strain AT300(pACYC184; empty vector) was 2 nmol (mg

Fig. 3. Influence of the NaCl concentration on the relative initial uptake activity of wild-type BetT. Cells of AT300(pAT3) were grown in half-strength M63 and the indicated amounts of NaCl were added 5 min prior to choline. The initial uptake activity of cells exposed to 0.4 M NaCl was set as 1.0, corresponding to 181 nmol min⁻¹ (mg protein)⁻¹. The values given are the means ± SD of measurements from at least three separate cell cultures.

protein)⁻¹ (data not shown). This background of bound radioactivity, which was not influenced by the osmotic conditions or the length of the incubation time with choline, was therefore used as the value for accumulation at time zero. The values for initial rate of choline uptake reported in Table 4 and those used to make Figs 3 and 4, were the means of 10–25 measurements from two to four independent cell cultures.

The optimal NaCl concentration for activation of BetT was 0.4 M; the transport activity decreased gradually at lower (0.1–0.3 M) and higher (0.5–0.8 M) salt concentrations

Fig. 4. Time-dependent activation of choline transport activity of wild-type BetT by 0.4 M NaCl. Cells were grown in half-strength M63 and exposed to osmotic stress for 0–10 min before the addition of choline. The initial uptake activity of cells exposed to NaCl for 5 min was set as 1.0, corresponding to 181 nmol min⁻¹ (mg protein)⁻¹. The values given are the means ± SD of measurements from at least two separate cell cultures.

(Fig. 3). Furthermore, the highest transport rate was observed 5 min after the cells were exposed to osmotic upshock. After that, the initial transport rate decreased 30 % during the next 5 min (Fig. 4). The total activation of BetT by incubation with 0.4 M NaCl for 5 min was nearly tenfold; i.e. the initial transport rate increased from 19 to 181 nmol min⁻¹ (mg protein)⁻¹ (Table 4). Cells that were activated with 0.55 M sucrose (total osmolality 760 mosmol kg⁻¹), in a transport assay medium without NaCl, displayed similar transport activity, i.e. 200 ± 29 nmol min⁻¹ (mg protein)⁻¹. Thus, the activation of BetT depended on osmotic stress and not on NaCl per se.

Transport properties of BetT mutant proteins

In Table 4, BetT mutants are given numbers which correspond to the remaining number of residues in BetT. The proteins were expressed in strain AT300 from pAT3-derived plasmids and activated with 0.4 M NaCl, as described above.

C-terminally truncated BetT proteins with deletions up to 101 residues conferred non-activated transport of choline, which in most cases was similar to that of the wild-type (Table 4). This background activity allowed us to measure their ability to be osmotically activated. A deletion of one (BetT676) or two (BetT675) residues did not influence the osmotic activation, which remained about tenfold, while deletions of six (BetT671) or 12 (BetT665) residues had a pronounced effect on the protein's ability to be activated. BetT mutants with deletions of 18–101 residues did not display any osmotic activation.

The six C-terminal residues of BetT are Val-Met-Phe-Pro-Asp-Ala. By *in vitro* mutagenesis, we made three BetT mutants in which two or three of these amino acids were changed. The substitutions in BetT677-M673A-F674A and BetT677-F764A-P675A-D676G were particularly detrimental to the process of osmotic activation, whereas the substitutions in BetT677-V672A-M673A caused effects that were similar to the deletion of the six C-terminal residues (Table 4).

As a control of the measurements of the initial transport activity, we measured the steady-state level of choline accumulation in the cells expressing the wild-type and the mutant BetT proteins. In general, there was a good correlation between the measurements; i.e. only the BetT proteins that displayed osmotic activation provoked a noticeably higher steady-state level of choline accumulation in the presence of 0.4 M NaCl than in the absence of salt (Table 4).

Kinetic analysis of wild-type BetT and BetT677-M673A-F674A were performed with cells grown and tested in M63 with 0.3 M NaCl. The observed K_m values were practically the same, i.e. 3 ± 1 μM. This is similar to the K_m value of 8 μM determined previously for cells carrying the *bet* genes on the chromosome (Styrvold *et al.*, 1986). Due to low

activities, we were unable to measure any K_m values for cells that had not been activated with NaCl. We also tested if BetT665 (Δ12) could be more activated by applying other concentrations of NaCl (0.2–0.8 M) or longer activation times (up to 20 min), but the results were negative (data not shown).

DISCUSSION

Our conclusion, from the present analyses of 36 PhoA and 15 LacZ fusions at 37 different sites in BetT, is that BetT has the predicted membrane topology of the BCCT family. Besides having 12 membrane-spanning regions, BetT has a small N-terminal domain of about 12 residues and a large C-terminal domain of about 180 residues, both situated in the cytoplasm.

It is now well established that bacterial osmosensors of many kinds sense the osmotic conditions in the cytoplasm, e.g. the transporters ProP of *E. coli* (Culham *et al.*, 2003), BetP of *C. glutamicum* (Rübenhagen *et al.*, 2001) and OpuA of *L. lactis* (van der Heide *et al.*, 2001), and the sensor kinases KdpD (Jung *et al.*, 2000) and EnvZ of *E. coli* (Jung *et al.*, 2001). We observed that BetT mutants carrying C-terminal deletions of 6, 12 or 18 amino acid residues displayed a gradual loss in osmotic activation of choline transport. Since the deletion mutants retained the transport activity at low osmolality, these data indicated that the C-terminal domain of 180 residues is involved in the osmoregulation of BetT rather than being necessary only for catalytic activity. The deletions of increasing lengths either eliminated a functional part or led to a misfolding of the entire domain, rendering it insensitive to osmotic stress. Similarly, we assume that the detrimental effects on osmotic activation by alanine and glycine substitutions within the last two to six C-terminal residues may be caused by increased flexibility of the tail, which again caused instability of the domain. Similar conclusions were reached by Schiller *et al.* (2006), who have identified a glutamate residue in the C-terminal end of BetP that is critical for osmosensing by its impact on the folding of this domain. Presumably, the C-terminal domain senses the ionic conditions in the cytoplasm and activates the transporter.

BetT mutants carrying C-terminal deletions of 18–101 residues were permanently locked in a low-activity, non-inducible, state. However, the opposite effect of short C-terminal deletions has been reported for the sodium-driven BetP transporter of *C. glutamicum*, i.e. these truncated transporters are permanently locked in an active state *in vivo* (Peter *et al.*, 1998; Morbach & Krämer, 2003). In this respect, ProP of *E. coli* may seem to behave more similarly to BetT than to BetP. For ProP, it is reported that an amino acid substitution (Arg-488-Ile) which hampers the formation of an antiparallel coiled-coil structure in its C-terminal cytoplasmic domain causes the transporter to be less sensitive to and only transiently activated by osmotic stress *in vivo* (Culham *et al.*, 2000; Zoetewey *et al.*, 2003). It is

also noteworthy that the *E. coli* CaiT transporter of the BCCT family has a deduced C-terminal extension of only ten residues. This transporter, which serves as an L-carnitine/ γ -butyrobetaine antiporter in anaerobic respiration, is not osmotically activated (Jung *et al.*, 2002). Since both BetP (Ziegler *et al.*, 2004) and CaiT (Vinothkumar *et al.*, 2006) form trimers in the membrane, it seems likely that BetT also has a trimeric structure. However, by analogy to CaiT, it seems unlikely that the large C-terminal extensions of BetP and BetT are required for the oligomerization.

The transport activity of wild-type BetT peaked 5 min after spectinomycin-treated cells were subjected to an osmotic upshock. It is therefore tempting to speculate that this was the time when the conditions in the cytoplasm were most favourable for BetT activation. In *S. meliloti*, it takes 8–10 min to fully activate glycine betaine uptake by BetS. However, unlike BetT, the transport rate of BetS remains constant for at least 3 h (Boscari *et al.*, 2002). Other transporters are fully activated faster than BetT, e.g. BetP reaches 90 % of maximum activity within 30 s after osmotic shock (Farwick *et al.*, 1995), whereas ProP of *E. coli* has an activation half-time of about 1 min (Milner *et al.*, 1988).

The LacZ fusion linked to Lys-12 displayed a particularly high β -galactosidase activity. The reason may be that this fusion produced a soluble protein. An additional explanation is that the region of *betT* encoding amino acid residues 7–30 may form a stem-loop structure of 29 bp with only two mismatches in mRNA (Lamark *et al.*, 1991). This putative structure, which may be involved in the regulation of BetT expression, was present in all fusions except the Lys-12 fusion. A similar reduction in the expression level of CodB–LacZ hybrid proteins of *E. coli* has previously been observed for fusion points which are situated after a weak transcriptional terminator (Danielsen *et al.*, 1995).

It is also noteworthy that our analysis of BetT–LacZ fusions showed that the *betT* promoter itself (without BetI) was down-regulated about tenfold in cells grown in LB (410 mosmol kg⁻¹) compared with cells grown in half-strength M63 (160 mosmol kg⁻¹) (Table 3). This aspect of *betT* regulation, which is not linked to hyperosmotic stress, has not been revealed previously.

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