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Identification of osmo-dependent and osmo-independent betaine-choline-carnitine transporters in *Acinetobacter baumannii*: role in osmostress protection and metabolic adaptation

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Summary

***Acinetobacter baumannii* is outstanding for its ability to cope with low water activities and therefore its adaptation mechanism to osmotic stress. Here we report on the identification and characterization of five different secondary active compatible solute transporters, belonging to the betaine-choline-carnitine transporter (BCCT) family. Our studies revealed two choline-specific and three glycine betaine-specific BCCTs. Activity of the BCCTs was differentially dependent to the osmolality: one choline and one betaine transporter were osmostress-independent. Addition of choline to resting cells of *Acinetobacter* grown in the presence of the co-substrate choline or with phosphatidylcholine as sole carbon source led to ATP synthesis in the wild type but not in the BCCT quadruple mutant. This indicates that the BCCTs are essential to transport the energy substrate choline. The role of the different BCCTs in osmostress resistance and in metabolic adaptation of *A. baumannii* to the human host is discussed.**

Introduction

The opportunistic human pathogen *Acinetobacter baumannii* is an emerging threat in healthcare institutions worldwide (Villegas and Hartstein, 2003; Antunes *et al*., 2014; Wong *et al*., 2017; Harding *et al*., 2018). The success of *A. baumannii* in clinical environments is based on its outstanding potential to adapt to extreme environments and to persist in the host (Allen and Green, 1987; Dijkshoorn *et al*., 1987; Dijkshoorn *et al*., 2007; Perez *et al*., 2007; Towner, 2009; Howard *et al*., 2012; Roca *et al*., 2012; Lee *et al*., 2017). The steadily increasing number of multi-resistant strains has led the World Health Organization to place carbapenem-resistant *Acinetobacter* (together with carbapenem-resistant *Pseudomonas aeruginosa* and carbapenem-resistant, ESBL-producing *Enterobacteriaceae*) on position 1 of the list of priority pathogens for which new antibiotics are urgently needed (World Health Organization, 2017; Tacconelli *et al*., 2018).

The adaptation of the Gram-negative *A. baumannii* to extreme dry and salty environments is based on the outstanding feature to cope with low water activities and osmotic stress (Breisch *et al*., 2018, Jawad *et al*., 1996, Wendt *et al*., 1997, Zeidler *et al*., 2017, Zeidler and Müller, 2018, Zeidler *et al*., 2018, Zeidler and Müller, 2019). These features also allow *A. baumannii* to persist on dry surfaces in clinical environments for weeks or even month. The increase of environmental osmolality due to water evaporation or the presence of osmotically active substances, such as salts or sugars, leads to a loss of water and thus, a reduction of the cellular turgor pressure. This can finally cause cell death, if no counter measures are taken (Wood, 1999; Bremer and Krämer, 2019). Living cells have developed two principal strategies to circumvent the dramatic consequences of water loss (Record Jr. *et al*., 1998; Wood, 1999). The first is the 'salt-in-cytoplasm' strategy which implies the accumulation of inorganic ions, such as K^+ and Cl^-, to restore the turgor (Heller *et al*., 1998; Roeßler and Müller, 2001). This strategy is typical for some halophilic archaea (*Halobacteria*) and a few moderately halophilic bacteria such as *Halanaerobiales* (Oren, 1999; Oren, 2008). The vast majority of bacteria cope with osmostress by the accumulation or synthesis of compatible solutes (Galinski, 1995; Wood *et al*., 2001; Pflüger and Müller, 2004; Bremer and Krämer, 2019). Compatible solutes are organic osmolytes promoting the proper folding of proteins and protein complexes without affecting cellular functions even at high concentrations (Burg and Ferraris, 2008; Empadinhas and da Costa, 2008). The spectrum

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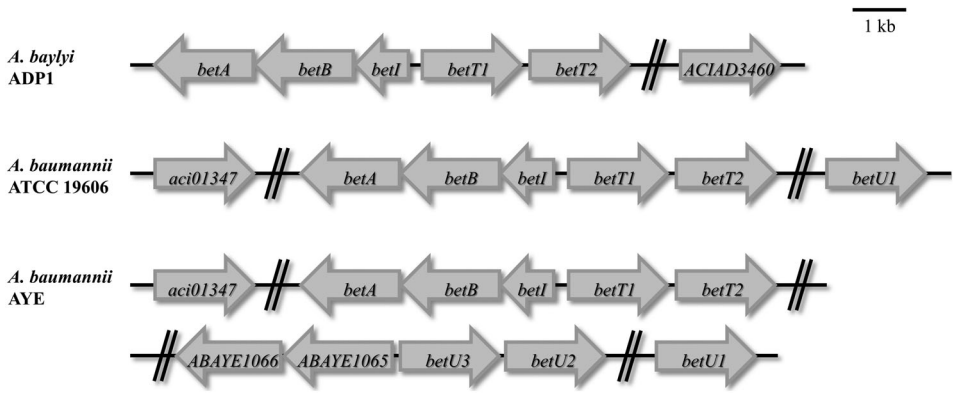

Fig. 1. Genetic organization of the BCCT gene loci in *A. baylyi*, *A. baumannii* ATCC 19606 and *A. baumannii* AYE. For further information, see text.

of compatible solutes is rather limited and can be assigned to two major groups; (i) sugars and polyols and (ii) α and β-amino acids and their derivatives including methylamines (Kempf and Bremer, 1998; Roeßler and Müller, 2001).

Like other bacteria, *A. baumannii* accumulates compatible solutes in response to osmostress. The major compatible solutes synthesized are glutamate and the unusual solute mannitol (Zeidler *et al*., 2018). Moreover, small amounts of trehalose were also detected (Zeidler *et al*., 2017). These three compatible solutes were also found in the related non-pathogenic soil bacterium *Acinetobacter baylyi* during growth in the presence of high salt (Sand *et al*., 2013; Zeidler *et al*., 2017). If present in the medium, *A. baylyi* takes up glycine betaine and the synthesis of glutamate, mannitol and trehalose is turned off (Sand *et al*., 2013). Uptake of glycine betaine is mediated by the secondary active transporter ACIAD3460, a member of the BCCT family (Sand *et al.*, 2011). This is consistent with the finding that uptake of solutes is preferred over *de novo* synthesis (Oren, 1999). *A. baylyi* can also take up choline, but choline does not serve as compatible solute *per se*. Instead it is oxidized by a choline dehydrogenase and a glycine betaine aldehyde dehydrogenase to the compatible solute glycine betaine. Interestingly, the genes encoding the dehydrogenases (*betA*, *betB*) are clustered with a gene encoding a potential regulator of the TetR-type (*betI*) and two genes transcribed divergently that encode BCCTs (Fig. 1). BetT2 was described as osmo-dependent and is most likely involved in uptake of choline as precursor for the compatible solute glycine betaine. In contrast to other bacteria such as *Escherichia coli* or *Bacillus subtilis*, *A. baylyi* can use choline not only as precursor of the compatible solute glycine betaine but also as additional energy source whereby choline is oxidized by BetA and BetB to glycine betaine; this metabolic feature is osmo-independent and requires a salt-independent transport system. Indeed, BetT1 was described as being osmo-independent and most likely accumulates choline as energy source (Sand *et al*., 2014; Scholz *et al*., 2016).

Although the synthesis of solutes in *A. baumannii* has been studied to some extent, relatively little is known about uptake systems for compatible solutes in *A. baumannii*. The BCCT Aci01347 in *A. baumannii* ATCC 19606 was annotated as choline transporter, but experimental analysis proved the assumption wrong, since Aci01347 was found to be essential for growth on carnitine as sole carbon and energy source (Breisch *et al*., 2018). Carnitine transport was osmolarity-independent which led to the suggestion that Aci01347 is important for growth on carnitine, which is abundant in animals and in the human host (Meadows and Wargo, 2015; Breisch *et al*., 2018). This points to an essential role of BCCTs not only in osmoadaptation but also in pathobiology of *A. baumannii*.

Since transporters for compatible solutes have not yet been described in *A. baumannii*, we searched the genome of the type strain ATCC 19606 and the multiresistant *A. baumannii* strain AYE for potential solute transporters and analysed their function biochemically. Their cellular function is discussed.

Results

Identification of BCCT encoding genes in the genome of A. baumannii *ATCC 19606 and AYE*

In previous studies, we reported that *aci01347* of *A. baumannii* ATCC 19606 encodes an osmo-independent carnitine/choline transporter of the BCCT family (Breisch *et al*., 2018). To search for further BCCTs in *A. baumannii* ATCC 19606 we performed a whole genome search. This led to the detection of three genes encoding BCCTs such as HMPREF0010_01463 (*betT1*), HMPREF0010_01464 (*betT2*) and HMPREF0010_03536 (*betU1*). HMPREF0010_01463 (*betT1*) and HMPREF0010_01464 (*betT2*) are clustered with the genes encoding a potential choline dehydrogenase (HMPREF0010_01460, *betA*) and a

betaine aldehyde dehydrogenase (HMPREF0010_01461, *betB*) and a potential transcriptional regulator (HMPREF0010_01462, *betI*) (Fig. 1). *betT1* encodes a 540-amino-acid protein with a deduced molecular mass of 58 kDa whereas *betT2* encodes a 688-amino-acid protein with a deduced molecular mass of 78 kDa. BetT1 and BetT2 of ATCC 19606 exhibit highest identities and similarities to the choline transporters BetT1 and BetT2 of *A. baylyi* (83% identity/97% similarity and 87% identity/96% similarity, respectively). BetT1 and BetT2 are 43% identical/78% similar to each other. Therefore, we conclude that *A. baumannii* strain ATCC 19606 is also able to take up choline and to use it twofold, like *A. baylyi*: as a precursor for the compatible solute glycine betaine and as an energy source (Scholz *et al*., 2016). The same holds true for strain AYE, which also has choline oxidation genes as well as *betT1* and *betT2* genes (Fig. 1). The similarities of the predicted proteins to the proteins of *A. baylyi* are in the range as for strain ATCC 19606 (83% identity/97% similarity for BetT1 and 87% identity/96% similarity for BetT2, respectively and 43% identity/78% similarity to each other).

In addition to the BCCTs BetT1 and BetT2 involved in choline metabolism, *Acinetobacter* strains have a BCC-type glycine betaine transporter (ACIAD3460 or BetU1 in *A. baylyi*). BetU1 of strain ATCC 19606 is 655 amino acids long with a deduced molecular mass of 74 kDa and is 77% identical and 94% similar to ACIAD3460 from *A. baylyi*. Same holds true for BetU1 of strain AYE, which also shows 77% identity and 94% similarity to ACIAD3460 of *A. baylyi*.

In addition, AYE has a second gene cluster encoding two potential BCCTs. *betU2* and *betU3* both clustered with a potential choline monooxygenase gene ABAYE1065 and a potential glycine betaine aldehyde dehydrogenase gene ABAYE1066 (Fig. 1). *BetU2* and *betU3* encode 558- and 565-amino-acid proteins with a deduced molecular mass of 61 kDa and 62 kDa, respectively. BetU2 and BetU3 both show highest similarities to the secondary active glycine betaine transporter BetU of *E. coli* (39% identity/71% similarity and 37% identity/71% similarity, respectively). BetU2 and BetU3 are 51% identical/79% similar to each other.

The BCCTs are involved in osmostress protection in A. baumannii *ATCC 19606*

To get insights into the role of the BCCTs in osmostress adaptation in *A. baumannii* ATCC 19606, *betT1*, *betT2* and *betU1* were deleted in the Δ*aci01347* mutant *via* two consecutive homologous recombination and segregation events using SacB-encoding plasmids yielding a quadruple markerless *betT1*/*betT2*/*betU1*/*aci01347* mutant. The mutant was verified by DNA sequencing. Growth of the quadruple mutant in mineral medium (MM) (μ = 1.2 h^{-1}) was comparable to growth of wild type cells (μ = 1.15 h^{-1}) (Fig. 2). In the presence of 500 mM NaCl, growth of the wild type and the quadruple mutant was slower with growth rates of 0.32 h^{-1} for both cultures (Fig. 2). The growth phenotype of the wild type could be rescued by addition of choline or glycine betaine (μ = 0.52 h^{-1} and μ = 0.56 h^{-1}), whereas growth of the quadruple mutant was not stimulated by choline (μ = 0.36 h^{-1}). Addition of glycine betaine to the quadruple mutant during growth at high salt did also not restore the wild type growth phenotype. However, after 9 h of slow growth (μ = 0.2 h^{-1}) the growth rate increased to μ = 0.47 h^{-1}. These results suggest that glycine betaine and choline function as osmoprotectants in *A. baumannii* ATCC 19606 and that at least one of the potential BCCTs BetT1, BetT2 or BetU1 is essential for choline or glycine

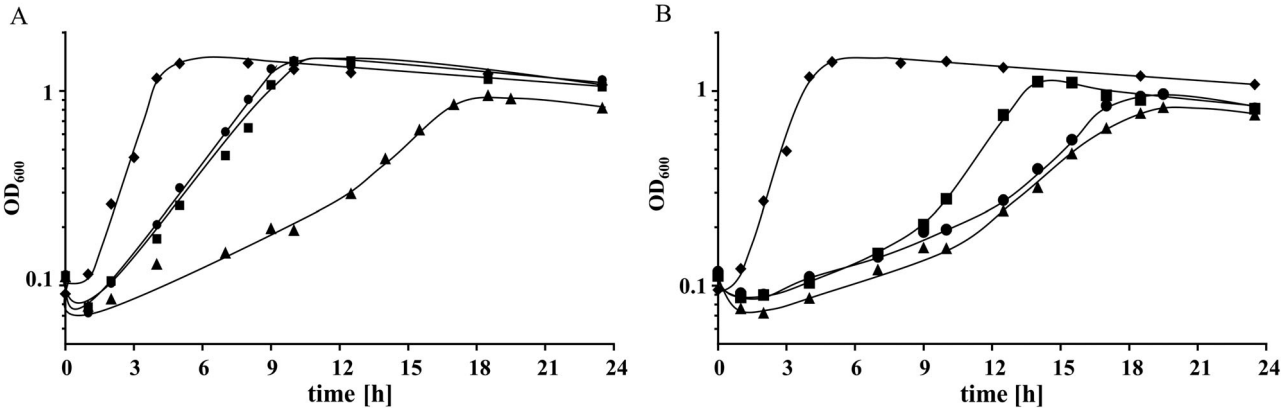

Fig. 2. Role of BCCTs in choline or glycine betaine dependent osmostress protection of *A. baumannii* ATCC 19606.
A. *A. baumannii* ATCC 19606 wild type was grown in MM with 20 mM NaAc (♦) in the presence of 500 mM NaCl (▲), 500 mM NaCl +1 mM choline (●) or 500 mM NaCl + glycine betaine (■).
B. *A. baumannii* BCCT quadruple mutant was grown in MM with 20 mM NaAc (♦) in the presence of 500 mM NaCl (▲), 500 mM NaCl +1 mM choline (●) or 500 mM NaCl + glycine betaine (■).
The data shown are representative data from one out of three comparable biological replicates.

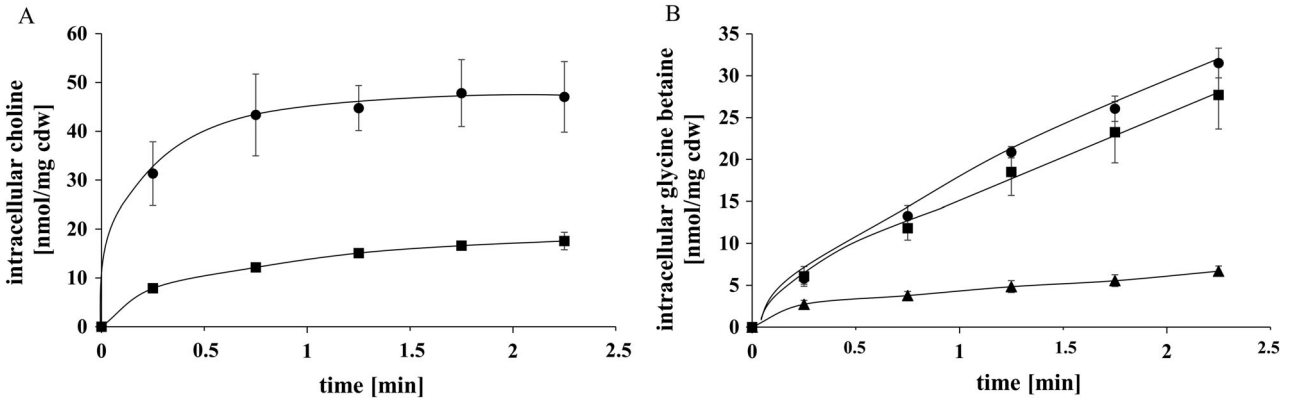

Fig. 3. Transport of choline or glycine betaine in *E. coli* MKH13 expressing *betT1*, *betT2*, *betU1*, *betU2 or betU3*. Cells were grown in LB medium, cell suspensions were generated and choline or glycine betaine uptake was analysed at an external osmolality of 0.2 osmol/kg.
A. [^{14}C]-choline uptake by *E. coli* MKH13 expressing *betT1* (■) or *betT2* (●) was started by adding 500 μM [^{14}C]-choline (0.1 μCi). Each value is the mean of ±S.E.M. of at least three independent measurements.
B. [^{14}C]-glycine betaine uptake by *E. coli* MKH13 expressing *betU1* (▲), *betU2* (■) or *betU3* (●) was started by adding 500 μM [^{14}C]-glycine betaine (0.1 μCi). Each value is the mean of ±S.E.M. of at least three independent measurements.

betaine uptake in ATCC 19606 under high salt conditions. The increased growth rate of the quadruple mutant after 9 h of growth in the presence of high salt and glycine betaine suggests that additional primary or secondary active glycine betaine transporters are induced under these conditions mediating the uptake of the compatible solute glycine betaine thereby promoting osmostress adaptation.

BetT1 and BetT2 are choline transporters and BetU1 is a glycine betaine transporter

To address the substrate specificities of BetT1, BetT2 and BetU1 the proteins were heterologously produced in *E. coli* MKH13 (Haardt *et al*., 1995) using the pBAD/HisA vector system. *E. coli* MKH13 lacks the compatible solute transporters PutP, ProP and ProU, which makes this strain a perfect host for *in vivo* studies of compatible solute transporters. Transport studies were performed with labelled [^{14}C]-choline or [^{14}C]-glycine betaine. These studies revealed that BetT1 and BetT2 both transport choline but differ significantly in initial choline uptake rates, such for BetT1 uptake rates of 5.3 nmol/min·mg dry weight were detected whereas BetT2 takes up choline with a rate of 7.2 nmol/min·mg dry weight (Fig. 3A). Moreover, the final accumulation factors differed between BetT1 and BetT2 by a factor of four (17.6 nmol/mg dry weight and 47 nmol/mg dry weight, respectively) (Fig. 4). BetU1 did not catalyse choline transport (data not shown) but catalyses glycine betaine uptake with an uptake rate of 1.9 nmol/min·mg dry weight (Fig. 3B). BetU1 had the smallest transport capacity and accumulated glycine betaine only to 6.7 nmol/mg dry weight (Fig. 4). *E. coli* MKH13 did not accumulate [^{14}C]-glycine betaine or [^{14}C]-choline (data not shown). These data are consistent with the hypothesis that BetT1 and BetT2 mediate choline uptake whereas BetU1 mediates glycine betaine uptake in *A. baumannii* ATCC 19606. This is underlined by the high similarities of BetT1 and BetT2 to choline transporters and BetU1 to the glycine betaine transporter of *A. baylyi*.

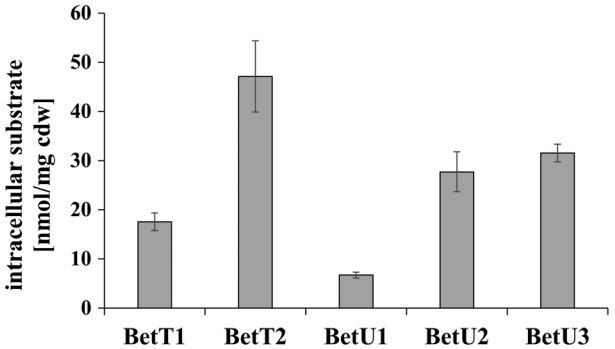

Fig. 4. Choline or glycine betaine accumulation of *E. coli* MKH13 strains expressing *betT1*, *betT2*, *betU1*, *betU2* and *betU3*. Cells were grown in LB medium, cell suspensions were generated and choline or glycine betaine uptake was analysed at an external osmolality of 0.2 osmol/kg. Uptake was started by adding 500 μM [^{14}C]-choline (0.1 μCi; BetT1, BetT2) or 500 μM [^{14}C]-glycine betaine (0.1 μCi; BetU1, BetU2, BetU3) and accumulation factors were determined after 2.5 min. Each value is the mean of ±S.E.M. of at least three independent measurements.

BetU2 and BetU3 are glycine betaine transporters

The high similarities of the two BCCTs BetU2 and BetU3 of AYE to secondary active glycine betaine transporters suggest that these two transporters mediate glycine betaine uptake. To address the role of BetU2 and BetU3 in

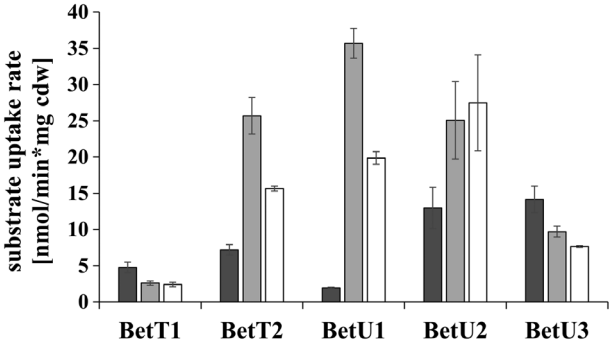

Fig. 5. Activity of BetT1, BetT2, BetU1, BetU2 and BetU3 under different external osmolalities. *E. coli* MKH13 expressing *betT1*, *betT2*, *betU1*, *betU2* or *betU3* were grown in LB medium, cell suspensions were generated and transport was analysed in KPi-buffer (25 mM, pH 7.5) with an adjusted OD_{600} of 1.5 at external osmolalities of 0.2 osmol/kg (dark grey), 0.6 osmol/kg (light grey) and 0.8 osmol/kg (white). Solute uptake was started by adding 500 μM [^{14}C]-choline (0.1 μCi; BetT1, BetT2) or 500 μM [^{14}C]-glycine betaine (0.1 μCi; BetU1, BetU2, BetU3). Each value is the mean of ±S.E.M. of at least three independent measurements.

glycine betaine uptake both transporters were heterologously produced in *E. coli* MKH13 and uptake studies were performed with radioactively labelled [^{14}C]-glycine betaine (Fig. 3B). Cells producing BetU2 and BetU3 accumulated [^{14}C]-glycine betaine with a rate of 12.9 nmol/min·mg dry weight and 14.2 nmol/min·mg dry weight, respectively (Fig. 3B), whereas *E. coli* MKH13 did not accumulate [^{14}C]-glycine betaine (data not shown). Both transporters have nearly the same transport capacity of 27.7 nmol/mg dry weight and 31.5 nmol/mg dry weight, respectively (Fig. 4). These findings lead to the conclusion that BetU2 and BetU3 mediate glycine betaine uptake.

BetT1 and BetU3 are osmo-independent BCCTs whereas BetT2, BetU1 and BetU2 are osmo-dependent

The finding that BetT1 and BetT2 mediate choline uptake and BetU1, BetU2 and BetU3 mediate glycine betaine uptake raised the question whether these BCCTs are involved in osmoadaptation. To address this question *E. coli* MKH13 pBAD/HisA_*betT1*, *E. coli* MKH13 pBAD/HisA_*betT2*, *E. coli* MKH13 pBAD/HisA_*betU1*, *E. coli* MKH13 pBAD/HisA_*betU2* and *E. coli* MKH13 pBAD/HisA_*betU3* were grown in complex medium, washed and resuspended with KPi-buffer containing an osmolality of 0.2 osmol/kg to an OD_{600} of 3 and diluted 1:1 in buffer with osmolalities of 0.2, 0.6 or 0.8 osmol/kg. As can be seen in Fig. 5, the activities of BetT2, BetU1 and BetU2 were clearly osmolality dependent. BetT2 showed highest choline and BetU1 highest glycine betaine transport activities of 25.6 nmol/min·mg dry weight and 35.7 nmol/min·mg dry weight, respectively, at 0.6 osmol/kg. BetU2 exhibited maximal glycine betaine transport activity of 27.5 nmol/min·mg dry weight at 0.8 osmol/kg. The presence of high salt led to a significant stimulation of the transport activities of these three transporters such as BetT2 was stimulated 3.5-fold and BetU1 and BetU2 were stimulated 18.8-fold and 2.1-fold, respectively. This is in line with the hypothesis that BetT2, BetU1 and BetU2 are osmo-dependent BCCTs. In contrast, the choline transport activity of BetT1 and the glycine betaine transport activity of BetU3 were not stimulated by high osmolality. Highest transport activities of 5.3 nmol/min·mg dry weight (BetT1) and 14.2 nmol/min·mg dry weight (BetU3) were detected at low osmolality (Fig. 5). The presence of high salt (0.8 osmol/kg) led to a twofold inhibition of the choline transport activity of BetT1 and a 1.9-fold inhibition of the glycine betaine transport activity of BetU3. These results lead to the conclusion that BetT1 is an osmo-independent choline and BetU3 is an osmo-independent glycine betaine transporter.

The BCCTs of A. baumannii *have different substrate affinities*

To analyse the substrate affinities of the different BCCTs, uptake studies in the presence of different choline or glycine betaine concentrations were performed under optimal osmolalities (0.2 osmol/kg for BetT1 and BetU3, 0.6 osmol/kg for BetT2 and BetU1 and 0.8 osmol/kg for BetU2). These studies revealed that the choline transporter BetT1 (Fig. 6A) and the glycine betaine transporter BetU2 (Fig. 6D) have very high substrate affinities (K_M values: 25.6 μM for BetT1 and 24.8 μM for BetU2) whereas the choline transporter BetT2 has a fivefold lower affinity to its substrate (K_M value: 129.2 μM, Fig. 6B) and the glycine betaine transporter BetU3 even a 10-fold lower affinity (K_M value: 237.2 μM) than BetT1 or BetU2 (Fig. 6E). The glycine betaine transporter BetU1 was found to have a high substrate affinity (K_M value of 64.5 μM, Fig. 6C).

BetT1 and BetT2 are highly specific for choline whereas BetU1, BetU2 and BetU3 mediate glycine betaine and proline betaine uptake

To analyse the substrate specificities of the BCCTs, competition experiments were performed in KPi-buffer under optimal osmolality conditions for each transporter. The uptake of [^{14}C]-choline or [^{14}C]-glycine betaine was followed in the presence of 50-fold excess of the competitors choline, glycine betaine, carnitine, ectoine, proline or proline betaine (Table 1). As expected, the addition of a 50-fold excess of unlabelled choline reduced the accumulation of labelled choline by BetT1 and BetT2 14.8-fold

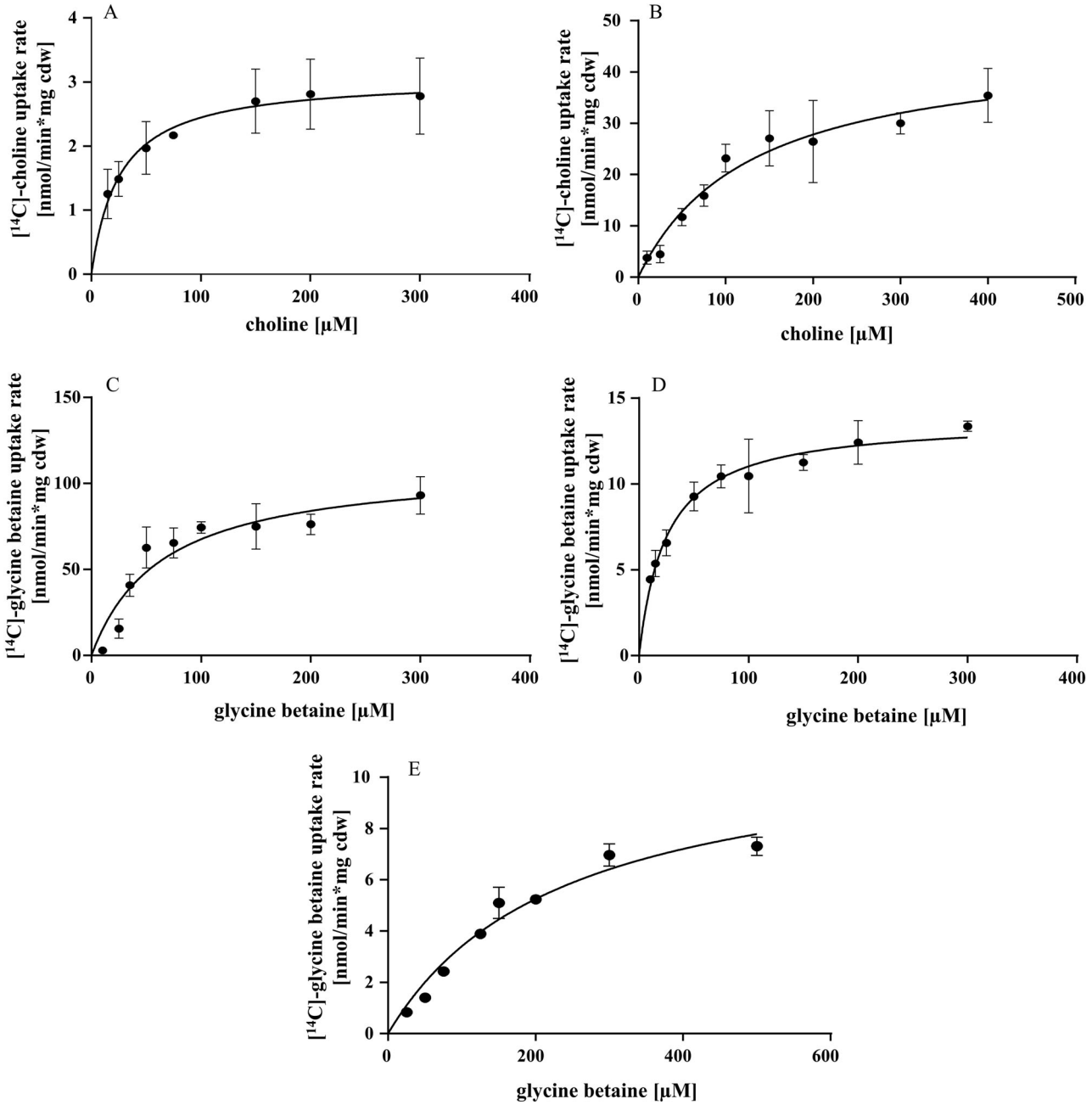

Fig. 6. Michealis Menten kinetics for BetT1, BetT2, BetU1, BetU2 and BetU3. Cells were grown in LB medium, cell suspensions were generated and transport was analysed in KPi-buffer (25 mM, pH 7.5) with an adjusted OD_{600} of 1.5 at optimal external osmolalities (0.2 osmol/kg: BetT1, BetT3; 0.6 osmol/kg: BetT2, BetU1; 0.8 osmol/kg: BetU2). BetT1 (A), BetT2 (B), BetU1 (C), BetU2 (D) and BetU3 (E) were analysed for the uptake of their preferred substrates. [^{14}C]-choline or [^{14}C]-glycine betaine (0.1 µCi) was added and non-labelled substrate in concentrations as indicated. The initial transport rates are plotted against the external solute concentration. Each value is the mean of ±S.E.M. of at least three independent measurements.

and 4.2-fold, respectively. Glycine betaine, carnitine, ectoine, proline and proline betaine had no effect on choline uptake by BetT1 and BetT2, demonstrating that BetT1 and BetT2 are choline specific. The addition of 50-fold excess of unlabelled glycine betaine reduced BetU1- and BetU2-mediated uptake of labelled glycine betaine 3.5-fold and 7.3-fold, respectively. Choline, carnitine, ectoine and proline had no effect on glycine betaine uptake by BetU1 and BetU2 whereas proline betaine led to a 3.1-fold and 29.3-fold inhibition of glycine betaine uptake, respectively.

Table 1. Effect of different competitors on solute uptake of *E. coli* MKH13 expressing *betT1*, *betT2*, *betU1*, *betU2* or *betU3*. Uptake of [^{14}C]-labelled solutes was followed in KPi-buffer (25 mM, pH 7,5) at optimal external osmolalities (0.2 osmol/kg for BetT1 and BetU3, 0.6 osmol/kg for BetT2 and BetU1, 0.8 osmol/kg for BetU2). Uptake was started by adding 10 μM [^{14}C]-choline (0.1 μCi; BetT1, BetT2) or 10 μM [^{14}C]-glycine betaine (0.1 μCi; BetU1, BetU2, BetU3) and 500 μM competitor to cell suspensions of the different recombinant *E. coli* MKH13 strains. The accumulated amount of [^{14}C]-choline or [^{14}C]-glycine betaine was analysed after 5 min of incubation. Negative controls were performed without competitor. Each value is the mean of ±S.E.M. of at least three independent measurements.

	[^{14}C]-choline or [^{14}C]-glycine betaine uptake [nmol/mg cdw] in the presence of different competitors						
	Control	Choline	Glycine betaine	Carnitine	Ectoine	Proline	Proline betaine
BetT1	5.9 ± 0.3	0.4 ± 0.03	6.1 ± 0.37	6.5 ± 0.49	6.2 ± 0.2	6.1 ± 0.32	5 ± 0.25
BetT2	14.3 ± 2.33	3.4 ± 0.05	16.8 ± 0.08	16.3 ± 0.32	16.9 ± 0.77	17.1 ± 0.37	16.7 ± 0.93
BetU1	18.3 ± 1.58	19.3 ± 0.27	5.2 ± 0.1	19.1 ± 1.13	18.3 ± 0.92	20.3 ± 1.23	5.9 ± 1.05
BetU2	17.6 ± 0.2	17.4 ± 0.37	2.4 ± 0.14	18 ± 0.16	17.5 ± 0.41	15.2 ± 0.18	0.6 ± 0.08
BetU3	2.7 ± 0.06	2.8 ± 0.05	2.4 ± 0.02	2.8 ± 0.24	2.8 ± 0.06	2.9 ± 0.15	1.0 ± 0.19

This suggests that BetU1 and BetU2 have narrow substrate specificities with glycine betaine and proline betaine. A 50-fold excess of unlabelled glycine betaine had no effect on BetU3-mediated glycine betaine uptake, whereas a 500-fold excess of unlabelled glycine betaine reduced glycine betaine uptake 2-fold (data not shown). Further, a 50-fold excess of unlabelled proline betaine reduced glycine betaine uptake 2.7-fold. However, the unaffected glycine betaine uptake of BetU3 in the presence of a 50-fold excess of unlabelled glycine betaine in contrast to the reduced glycine betaine uptake in the presence of same amounts of proline betaine indicates that proline betaine is even a better substrate for BetU3 than glycine betaine.

Oxidation of choline is a clear energetic benefit for A. baumannii

The data presented so far are in line with the hypothesis that *A. baumannii* ATCC 19606 and AYE use choline as an energy source. To address this hypothesis, cells were grown in MM, resting cells were prepared and the intracellular ATP level was analysed under different conditions. When cells were grown in the absence of choline, addition of choline to the cell suspension did not lead to an increase in intracellular ATP levels. However, when cells were grown in the presence of choline addition of choline led to an increase of the intracellular ATP content by nearly 100% (Fig. 7A), demonstrating choline-dependent energy conservation. The quadruple mutant did not synthesize ATP upon addition of choline, as expected, if the BCCTs are essential for choline uptake.

ATP synthesis coupled to choline metabolism was by a proton-dependent chemiosmotic mechanism. It was inhibited by the protonophore tetrachlorosalicylanilide (TCS) as well as by valinomycin + KCl, which also dissipate the electrochemical membrane potential across the cytoplasmic membrane (Heise *et al*., 1991). The electrochemical potential then drives the synthesis of ATP *via* the F_1F_0-type ATP synthase, as evident from the inhibition of ATP synthesis by the well-known F_1F_0-type ATP synthase inhibitor dicyclohexylcarbodiimide (DCCD) (Altendorf, 1977; Fillingame, 1997) and diethylstilbestrol (DES) (McEnery and Pedersen, 1986) (Fig. 7B).

Phosphatidylcholine induces choline dependent energy conservation

The major source of choline in host cells is the very abundant membrane lipid phosphatidylcholine (PC) which is the major lipid in human lung epithelial cells. To analyse the role of PC in choline-dependent energy conservation *A. baumannii* ATCC 19606 wild type was grown with PC as sole carbon and energy source. Addition of choline to the resting cells grown with PC led to an increase in ATP level by nearly 33.3% (Fig. 7A). This suggests that PC induces the choline dependent energy conservation pathway.

Discussion

A. baumannii is known for its unique capability to cope with osmotic stress as well as desiccation. The latter is suggested to play an important role in adaptation of *A. baumannii* to dry surfaces in hospital environments thereby favouring the survival and spread of *A. baumannii* in hospitals worldwide (Dijkshoorn *et al*., 2007). *A. baumannii* can cope with high osmolarities of up to 500 mM NaCl (Zeidler *et al*., 2017). This osmostress resistance might foster the survival of *A. baumannii* in high osmolaritiy host environments such as human blood (150 mM NaCl), intestinal lumen (300 mM NaCl) or humane urine (400 mM NaCl) (Chowdhury *et al*., 1996; Culham *et al*., 2001). The adaptation of *A. baumannii* ATCC 19606 to high salinities is mediated by the production of compatible solutes such as glutamate and mannitol and minor amounts of trehalose (Zeidler *et al*., 2017). Exogenous trehalose significantly supported

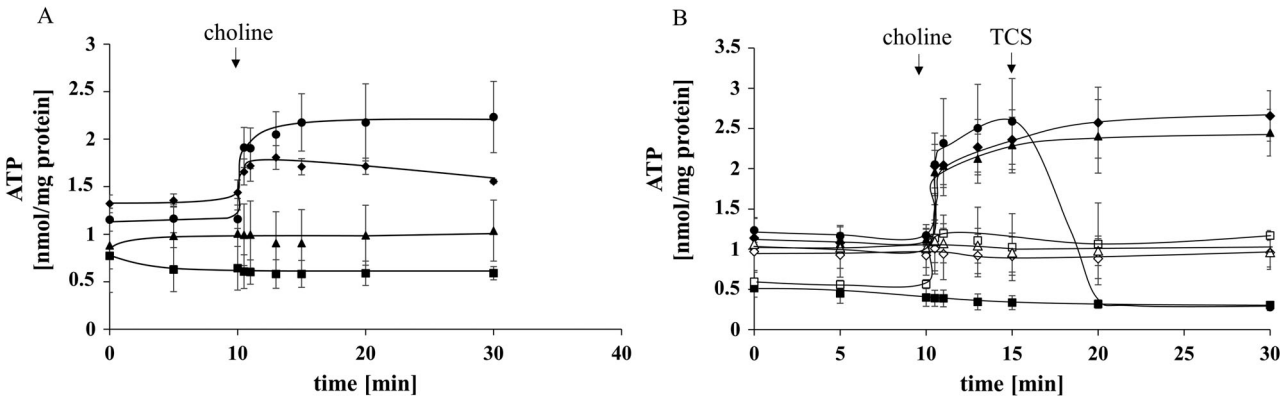

Fig. 7. Choline-dependent ATP synthesis in *A. baumannii* cell suspensions.
A. Cells were grown in MM with 12 mM succinate as carbon source and in the presence of 20 mM choline as additional energy source (wild type: ●, quadruple mutant: ▲) or with 0.5% PC as sole carbon and energy source (wild type: ♦) or in MM with 12 mM succinate as carbon and energy source in the absence of choline (wild type: ■). Cell suspensions were generated as described in materials and methods and ATP synthesis was started by addition of 10 mM choline after 10 min of preincubation of the cell suspension at 37°C. Samples were taken as indicated. Each value is the mean of ±S.E.M. of at least three independent measurements.
B. Cells were grown in MM with 12 mM succinate as carbon source and in the presence of 20 mM choline as additional energy source and cell suspensions were generated as described in materials and methods. ATP synthesis was started by addition of 10 mM choline after 10 min of preincubation of the cell suspensions at 37°C in the presence of 30 μM TCS (■), 30 μM valinomycin + 0.2 M KCl (□), 300 μM DES (◇), 300 μM DCCD (△), 30 μl 100% ethanol control (♦) or without inhibitor (▲). One suspension was preincubated for 10 min at 37°C in the absence of any inhibitor, ATP synthesis was started by the addition of 10 mM choline followed by the addition of 30 μM TCS 5 min after ATP synthesis was started (●). Each value is the mean of ±S.E.M. of at least three independent measurements.

viability of *A. baumannii* under desiccation stress, but trehalose is not taken up by primary active or secondary active transporters in *A. baumannii* (Zeidler and Müller, 2018). Uptake of solutes is generally preferred over *de novo* synthesis for energetic reasons (Oren, 1999).

Here we report on the identification of different BCCTs in *A. baumannii* ATCC 19606 and in the multiresistant *A. baumannii* strain AYE mediating the uptake of the compatible solute glycine betaine and its precursor choline. Mutant studies and transport assays provide clear evidence that two of the BCCTs mediate choline uptake whereas three are specific for glycine betaine. The transport activities of one choline (BetT1) and one glycine betaine transporter (BetU3) are osmostress independent, which leads to the suggestion that these BCCTs play a role beyond osmostress protection and suggest that they might be important for adaptation to choline- or glycine betaine-containing environments in the human host.

The glycine betaine and choline transporters of *A. baumannii* ATCC 19606 do not only differ in osmodependence but also exhibit significant differences in substrate affinities, such as K_M values ranging from ≈ 25 to 250 μM were estimated during uptake studies with recombinant *E. coli* strains producing distinct BCCTs. These affinities correlate with the physiological conditions during host infection such as the glycine betaine concentration in most animal fluids are ≈ 35 μM and the choline concentrations in serum are ≈ 8 μM (Melse-Boonstra *et al*., 2005). The finding that the K_M values of different choline- or glycine betaine-transporting BCCTs are in the range of the substrate concentrations in the host suggests that these BCCTs mediate choline or glycine betaine uptake in the human host.

In previous studies we have analysed the path of choline uptake and oxidation in the close relative *A. baylyi* (Sand *et al*., 2014; Scholz *et al*., 2016). Uptake of choline in *A. baylyi*, either in the presence or absence of salt is followed by a rapid oxidation of choline to glycine betaine by a conserved choline dehydrogenase (BetA) and a glycine betaine aldehyde dehydrogenase (BetB) (Scholz *et al*., 2016). The presence of homologues in the genome of *A. baumannii* ATCC 19606 together with our finding that choline leads to significant stimulation of growth in the presence of high salt suggests that this strain also oxidizes choline to the compatible solute glycine betaine. *A. baumannii* infections often occur in patients receiving mechanical ventilation by airway colonization of *A. baumannii* followed by pneumonia. The glycine betaine produced *via* choline oxidation might contribute to *A. baumannii* survival in the lung as it has already been shown for *P. aeruginosa* that glycine betaine production by the choline oxidase system increased the survival during murine lung infection although the mechanism of glycine betaine-dependent survival remains unknown (Wargo, 2013). Whether glycine betaine indeed contributes to the survival of *A. baumannii* during lung infection will be subject of future studies.

The presence of a second gene cluster potentially mediating choline oxidation in AYE is puzzling, especially the presence of the two additional glycine betaine

transporters BetU2 and BetU3. Glycine betaine produced from choline could be exported by BetU2 and BetU3, or BetU2 and BetU3 mediate the uptake of glycine betaine but the further fate of glycine betaine (reduction to choline) remains to be identified.

Our studies revealed that BCCTs mediated choline uptake leads to the synthesis of ATP and therefore an energetic benefit in choline rich environments. Analyses of the choline-dependent ATP levels in the presence of uncouplers and protonophores lead to the conclusion that the electrons derived from choline oxidation are funnelled through the respiratory chain creating an electrochemical gradient of protons across the cytoplasmic membrane which in turn drives ATP synthesis through an ATP synthase. The finding that growth of *A. baumannii* ATCC 19606 in the absence of choline did not lead to elevated ATP levels suggests that choline is required as inducer of the choline oxidation pathway and the choline transporters. This is consistent with the finding in *A. baylyi*, where in the absence of choline no *betAB* and *betT1*/*betT2* transcripts were detectable (Scholz *et al*., 2016).

The most abundant source of choline in the human host is the choline headgroup moiety of phosphatidylcholine (PC) and sphingomyelin (SM) which form 50% to 90% of the phospholipids in the outer leaflet of eukaryotic plasma membranes (Zachowski, 1993). Especially the membranes of lung epithelial cells are choline rich and consist of up to 70% PC (Keller and Ladda, 1979). Cleavage of PC or SM by phospholipases D (PLD) or phospholipases C (PLC) liberates choline and phosphorylcholine, respectively (Holbrook *et al*., 1991; Fiester *et al*., 2016). The release of phosphorylcholine from PC by PLC activity and subsequent phosphorylcholine phosphatase-mediated cleavage of phosphorylcholine to choline and phosphate has been well studied in the pathogen *P. aeruginosa* (Wargo *et al*., 2009). The haemolytic PLC of *P. aeruginosa* was found to be important for induction of pulmonary inflammation and for virulence in different eukaryotic model systems (Wargo *et al*., 2009). *A. baumannii* ATCC 19606 produces two PLCs and three PLDs, the latter have been identified to act in concerted manner as virulence factors (Stahl *et al*., 2015). The same holds true for the PLCs from *A. baumannii* ATCC 19606, such as PLC1 and PLC2 have been identified to be involved in lysing of different host cells (Fiester *et al*., 2016). Both, the PLCs and PLDs of *A. baumannii* ATCC 19606 are suggested to play an important role in adaptation to PC and SM rich membranous environments during human host infection. Recently we reported that *A. baumannii* ATCC 19606 can use PC as sole carbon and energy source and among the PC cleavage products glycerol, fatty acids and choline only fatty acids were found to serve as carbon and energy source (Stahl *et al*., 2015). Here we report that resting cells grown with PC as sole carbon and energy source exhibit choline-dependent elevated ATP levels. This suggests that the choline moiety of PC is liberated from PC thereby serving as inducer of the choline oxidation pathway. We propose that distinct phospholipases together with the osmo-independent choline transporter and the choline oxidation pathway are important for the gain of an energetic benefit supporting adaptation to the human host. The role of the different phospholipases in the gain of a choline-dependent energetic benefit of *A. baumannii* will be addressed in future studies.

Materials and methods

Bacterial strains and culture conditions

E. coli MKH13 was grown in LB medium (Bertani, 1951) at 37°C with 100 μg/ml ampicillin. *A. baumannii* strains were grown at 37°C in LB medium (Bertani, 1951) or in mineral medium (MM) that consists of 50 mM phosphate buffer, pH 6.8, and different salts (1 g NH_4Cl, 580 mg $MgSO_4 \times 7\ H_2O$, 100 mg KNO_3, 67 mg $CaCl_2 \times 2\ H_2O$, 2 mg $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$, 1 ml SL9 (per litre: 12.8 g Titriplex, 2 g $FeSO_4 \times 7\ H_2O$, 190 mg $CoCl_2 \times 6\ H_2O$, 122 mg $MnCl_2 \times 4\ H_2O$, 70 mg $ZnCl_2$, 36 mg $MoNa_2O_4 \times 2\ H_2O$, 24 mg $NiCl_2 \times 6\ H_2O$, 6 mg H_3BO_3, 2 mg $CuCl_2 \times H_2O$ per l medium; pH 6.5) (Tschech and Pfennig, 1984) and 20 mM sodium acetate as carbon source. Growth of *A. baumannii* under hyperosmotic conditions was analysed in MM with 500 mM NaCl and in the presence or absence of 1 mM choline or glycine betaine. Kanamycin of 50 μg/ml was added when appropriate. The growth experiments were repeated three times, and one representative experiment is shown. Growth curves were fitted manually and growth rates were determined with GraphPad Prism 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

Markerless mutagenesis of A. baumannii *ATCC 19606*

To generate a markerless triple mutant *Δaci01347*/*betT1*/*betT2*, 1500 bp upstream of *betT1* and 1500 bp downstream of *betT2* were amplified from *A. baumannii* ATCC 19606 genomic DNA (Primer pairs: Supporting Information Table S1) and cloned in the multiple cloning site of pBIISK_*sacB*/*kanR* (Stahl *et al*., 2015) using NotI and PstI. The resulting plasmid was transformed in the electrocompetent *A. baumannii Δaci01347* mutant (Breisch *et al*., 2018). For generation of electrocompetent cells, *A. baumannii* strains were grown in LB medium, harvested at $OD_{600} = 0.45$, washed four times in H_2O_{MQ}, and resuspended in 10% glycerol. Electroporation was performed at 2.5 kV, 200 Ω, and 25 μF. Transformants were selected on LB agar containing 50 μg/ml kanamycin and integrants were verified by PCR (Primer pairs: Supporting

Information Table S1). Segregation was induced by counter selection in LB with 10% sucrose for 18 h at 37°C and followed by plating on LB agar containing 10% sucrose. Segregation was verified by plating onto LB/kanamycin agar. Single colonies exhibiting kanamycin sensitivity were verified by PCR (Primer pairs: Supporting Information Table S1). A markerless quadruple mutant of *A. baumannii* ATCC 19606 was generated by amplification of 1500 bp up- and down-stream fragments of *betU3* (Primer pairs: Supporting Information Table S1) and cloned in the multiple cloning site of pBIISK_*sacB*/*kanR* (Stahl *et al*., 2015) using NotI and PstI. The resulting plasmid was transformed in the electrocompetent *A. baumannii Δaci01347*/*betT1*/*betT2* triple mutant and integrants were verified via PCR and sequencing (Primer pairs: Supporting Information Table S1). Segregation was induced by counter selection in low salt LB (5 g/l NaCl, 10 g/l tryptone and 10 g/l yeast extract) containing 5% sucrose to minimalize the osmotic stress and followed by plating onto low salt LB agar containing 5% sucrose. Segregants which had lost kanamycin resistance were verified by PCR and sequencing (Primer pairs: Supporting Information Table S1).

[^{14}C]-choline and [^{14}C]-glycine betaine uptake of recombinant E. coli *MKH13 strains*

To study the biochemical features of the different BCCTs the encoding genes were cloned into the expression vector pBAD/HisA and the plasmids were transformed into *E. coli* MKH13. The strains producing His-BetT1, His-BetT2, His-BetU1, His-BetU2 and His-BetU3 were cultivated at 37°C in LB medium containing ampicillin (100 μg/ml). Gene expression was induced at OD_{600} of 0.6–0.8 by adding 0.02% arabinose. After 2.5 h the cells were harvested and washed with 25 mM KPi buffer (pH 7.5) containing 100 mM NaCl and resuspended in the same buffer containing 30 mM glucose to an OD_{600} of 3. For uptake studies, cell suspensions were diluted 1:1 (OD_{600} = 1.5) with 25 mM KPi buffer (pH 7.5) containing 100 mM NaCl. KCl was used to adjust osmolalities of 0.2, 0.6 or 0.8 osmol/kg (OSMOMAT 3000; Gonotec GmbH, Berlin, Germany). Cells were incubated for 5 min at 37°C before the transport assays were started by the addition of 500 μM [^{14}C]-substrate (1 μCi). Samples (200 μl) were taken at indicated time points and the cells were separated from the medium by filtration through nitrocellulose filters (pore size: Ø = 0.45 μm) and washed with 20 volumes of 600 mM KPi buffer (pH 7.5). Filters were dried and dissolved in 8 ml scintillation solution (Rotizint^{R} eco plus; Carl-Roth GmbH, Karlsruhe, Germany) and radioactivity was determined by liquid scintillation counting.

For K_M and V_{max} determination, the concentrations of [^{14}C]-choline or [^{14}C]-glycine betaine were varied. The kinetic constants were plotted by Michaelis–Menten curve fitting of the uptake rates versus the substrate concentrations with GraphPad Prism 6.00 for windows (GraphPad Software, La Jolla, California, USA). For competition experiments, 10 μM of [^{14}C]-choline or [^{14}C]-glycine betaine and 500 μM of the given competitors were used. Intracellular [^{14}C]-choline or [^{14}C]-glycine betaine concentrations were analysed after 5 min of incubation at 37°C.

Detection of intracellular ATP levels

A. baumannii strains were grown in MM for 16 h at 37°C with 12 mM succinate as carbon source and in the presence or absence of 20 mM choline or for 40 h at 37°C with 0.5% PC as carbon source. Cells were harvested, washed in 1× PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137.9 mM NaCl, 8.1 mM Na_2HPO_4) and resuspended in the same buffer. The protein concentrations were determined by Schmidt *et al*. Ten-millilitre cell suspensions (1 mg protein/ml) were preincubated for 10 min at 37°C. ATP synthesis was induced by addition of choline to a final concentration of 20 mM. At time points indicated 400 μl samples were taken and mixed with 150 μl chilled 3 M perchloric acid. After 10 min of incubation at 4°C samples were neutralized with a saturated K_2CO_3 solution followed by the addition of 80 μl 400 mM TES buffer (pH 7.6) and 1 ml H_2O. After pelleting of the K_2CO_3 for 5 min at 17000 g and 4°C, 10 μl of the supernatant were mixed with 300 μl of determination buffer (5 mM Na_2HAsO_4, 4 mM $MgSO_4$, 20 mM glycylglycine) (Kimmich *et al*., 1975). ATP levels were determined by a luciferin-luciferase test (20 μl luciferin-luciferase, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) *via* monitoring the emitted light with a chemiluminometer (Junior LB 9509, Berthold Technologies, Bad Wildbach, Germany).

To analyse to effect of protonophores and ionophores on choline-dependent ATP generation the cell suspensions were preincubated for 10 min at 37°C in the presence of 30 μM TCS, 30 μM valinomycin and 0.2 M KCl, 300 μM DCCD, 300 μM DES or 30 μl 100% ethanol before ATP synthesis was induced by choline.

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Conflict of Interest

The authors declare no conflict of interest.

References

Allen, K.D., and Green, H.T. (1987) Hospital outbreak of multi-resistant *Acinetobacter anitratus*: an airborne mode of spread? *J Hosp Infect* **9**: 110–119.

Altendorf, K. (1977) Purification of the DCCD-reactive protein of the energy-transducing adenosine triphosphatase complex from *Escherichia coli*. *FEBS Lett* **73**: 271–275.

Antunes, L.C., Visca, P., and Towner, K.J. (2014) *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog Dis* **71**: 292–301.

Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**: 293–300.

Breisch, J., Waclawska, I., and Averhoff, B. (2018) Identification and characterization of a carnitine transporter in Acinetobacter baumannii. *Microbiology open* **6**: e00752.

Bremer, E., and Krämer, R. (2019) Responses of microorganisms to osmotic stress. *Annu Rev Microbiol* **73**: 313–334.

Burg, M.B., and Ferraris, J.D. (2008) Intracellular organic osmolytes: function and regulation. *J Biol Chem* **283**: 7309–7313.

Chowdhury, R., Sahu, G.K., and Das, J. (1996) Stress response in pathogenic bacteria. *J Biosci* **21**: 149–160.

Culham, D.E., Lu, A., Jishage, M., Krogfelt, K.A., Ishihama, A., and Wood, J.M. (2001) The osmotic stress response and virulence in pyelonephritis isolates of *Escherichia coli*: contributions of RpoS, ProP, ProU and other systems. *Microbiology* **147**: 1657–1670.

Dijkshoorn, L., Van Vianen, W., Degener, J.E., and Michel, M.F. (1987) Typing of *Acinetobacter calcoaceticus* strains isolated from hospital patients by cell envelope protein profiles. *Epidemiol Infect* **99**: 659–667.

Dijkshoorn, L., Nemec, A., and Seifert, H. (2007) An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* **5**: 939–951.

Empadinhas, N., and da Costa, M.S. (2008) Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. *Int Microbiol* **11**: 151–161.

Fiester, S.E., Arivett, B.A., Schmidt, R.E., Beckett, A.C., Ticak, T., Carrier, M.V., *et al*. (2016) Iron-regulated phospholipase C activity contributes to the cytolytic activity and virulence of *Acinetobacter baumannii*. *PLoS One* **11**: e0167068.

Fillingame, R.H. (1997) Coupling H+ transport and ATP synthesis in F1F0-ATP synthases: glimpses of interacting parts in a dynamic molecular machine. *J Exp Biol* **200**: 217–224.

Galinski, E.A. (1995) Osmoadaptation in bacteria. *Adv Microb Physiol* **37**: 272–328.

Haardt, M., Kempf, B., Faatz, E., and Bremer, E. (1995) The osmoprotectant proline betaine is a major substrate for the binding-protein-dependent transport system ProU of *Escherichia coli* K-12. *Mol Gen Genet* **246**: 783–786.

Harding, C.M., Hennon, S.W., and Feldman, M.F. (2018) Uncovering the mechanisms of *Acinetobacter baumannii* virulence. *Nat Rev Microbiol* **16**: 91–102.

Heise, R., Reidlinger, J., Müller, V., and Gottschalk, G. (1991) A sodium-stimulated ATP synthase in the acetogenic bacterium *Acetobacterium woodii*. *FEBS Lett* **295**: 119–122.

Heller, R., Höller, C., Süssmuth, R., and Gundermann, K.O. (1998) Effect of salt concentration and temperature on survival of *Legionella pneumophila*. *Lett Appl Microbiol* **26**: 64–68.

Holbrook, P.G., Pannell, L.K., and Daly, J.W. (1991) Phospholipase D-catalyzed hydrolysis of phosphatidylcholine occurs with P-O bond cleavage. *Biochim Biophys Acta* **1084**: 155–158.

Howard, A., O'Donoghue, M., Feeney, A., and Sleator, R.D. (2012) *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* **3**: 243–250.

Jawad, A., Heritage, J., Snelling, A.M., Gascoyne-Binzi, D. M., and Hawkey, P.M. (1996) Influence of relative humidity and suspending menstrua on survival of *Acinetobacter* spp. on dry surfaces. *J Clin Microbiol* **34**: 2881–2887.

Keller, G.H., and Ladda, R.L. (1979) Quantitation of phosphatidylcholine secretion in lung slices and primary cultures of rat lung cells. *Proc Natl Acad Sci U S A* **76**: 4102–4106.

Kempf, B., and Bremer, E. (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch Microbiol* **170**: 319–330.

Kimmich, G.A., Randles, J., and Brand, J.S. (1975) Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal Biochem* **69**: 187–206.

Lee, C.R., Lee, J.H., Park, M., Park, K.S., Bae, I.K., Kim, Y. B., *et al*. (2017) Biology of *Acinetobacter baumannii*: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. *Front Cell Infect Microbiol* **7**: 55.

McEnery, M.W., and Pedersen, P.L. (1986) Diethylstilbestrol. A novel F0-directed probe of the mitochondrial proton ATPase. *J Biol Chem* **261**: 1745–1752.

Meadows, J.A., and Wargo, M.J. (2015) Carnitine in bacterial physiology and metabolism. *Microbiology* **161**: 1161–1174.

Melse-Boonstra, A., Holm, P.I., Ueland, P.M., Olthof, M., Clarke, R., and Verhoef, P. (2005) Betaine concentration as a determinant of fasting total homocysteine concentrations and the effect of folic acid supplementation on betaine concentrations. *Am J Clin Nutr* **81**: 1378–1382.

Oren, A. (1999) Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63**: 334–348.

Oren, A. (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Systems* **4**: 2.

Perez, F., Hujer, A.M., Hujer, K.M., Decker, B.K., Rather, P. N., and Bonomo, R.A. (2007) Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **51**: 3471–3484.

Pflüger, K., and Müller, V. (2004) Transport of compatible solutes in extremophiles. *J Bioenerg Biomembr* **36**: 17–24.

Record, M.T., Jr., Courtenay, E.S., Cayley, D.S., and Guttman, H.J. (1998) Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem Sci* **23**: 143–148.

Roca, I., Espinal, P., Vila-Farres, X., and Vila, J. (2012) The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. *Front Microbiol* **3**: 148.

Roeßler, M., and Müller, V. (2001) Chloride dependence of glycine betaine transport in *Halobacillus halophilus*. *FEBS Lett* **489**: 125–128.

Sand, M., Mingote, A.I., Santos, H., Müller, V., and Averhoff, B. (2013) Mannitol, a compatible solute synthesized by *Acinetobacter baylyi* in a two-step pathway including a salt-induced and salt-dependent mannitol-1-phosphate dehydrogenase. *Environ Microbiol* **15**: 2187–2197.

Sand, M., Stahl, J., Waclawska, I., Ziegler, C., and Averhoff, B. (2014) Identification of an osmo-dependent and an osmo-independent choline transporter in *Acinetobacter baylyi*: implications in osmostress protection and metabolic adaptation. *Environ Microbiol* **16**: 1490–1502.

Sand, M., de Berardinis, V., Mingote, A., Santos, H., Göttig, S., Müller, V., & Averhoff, B. (2011). Salt adaptation in *Acinetobacter baylyi*: identification and characterization of a secondary glycine betaine transporter. *Arch Microbiol*, **193**: 723–730.

Scholz, A., Stahl, J., de Berardinis, V., Müller, V., and Averhoff, B. (2016) Osmotic stress response in *Acinetobacter baylyi*: identification of a glycine-betaine biosynthesis pathway and regulation of osmoadaptive choline uptake and glycine-betaine synthesis through a choline-responsive BetI repressor. *Environ Microbiol Rep* **8**: 316–322.

Stahl, J., Bergmann, H., Göttig, S., Ebersberger, I., and Averhoff, B. (2015) *Acinetobacter baumannii* virulence is mediated by the concerted action of three phospholipases D. *PLoS One* **10**: e0138360.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., *et al*. (2018) Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* **18**: 318–327.

Tschech, A., and Pfennig, N. (1984). Growth yield increase linked to caffeate reduction in Acetobacterium woodii. *Arch Microbiol*, **137**: 163–167.

Towner, K.J. (2009) *Acinetobacter*: an old friend, but a new enemy. *J Hosp Infect* **73**: 355–363.

Villegas, M.V., and Hartstein, A.I. (2003) *Acinetobacter* outbreaks, 1977-2000. *Infect Control Hosp Epidemiol* **24**: 284–295.

Wargo, M.J. (2013) Choline catabolism to glycine betaine contributes to *Pseudomonas aeruginosa* survival during murine lung infection. *PLoS One* **8**: e56850.

Wargo, M.J., Ho, T.C., Gross, M.J., Whittaker, L.A., and Hogan, D.A. (2009) GbdR regulates *Pseudomonas aeruginosa plcH* and *pchP* transcription in response to choline catabolites. *Infect Immun* **77**: 1103–1111.

Wendt, C., Dietze, B., Dietz, E., and Rüden, H. (1997) Survival of *Acinetobacter baumannii* on dry surfaces. *J Clin Microbiol* **35**: 1394–1397.

Wong, D., Nielsen, T.B., Bonomo, R.A., Pantapalangkoor, P., Luna, B., and Spellberg, B. (2017) Clinical and pathophysiological overview of *Acinetobacter* infections: a century of challenges. *Clin Microbiol Rev* **30**: 409–447.

Wood, J.M. (1999) Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev* **63**: 230–262.

Wood, J.M., Bremer, E., Csonka, L.N., Kraemer, R., Poolman, B., van der Heide, T., and Smith, L.T. (2001) Osmosensing and osmoregulatory compatible solute accumulation by bacteria. *Comp Biochem Physiol A Mol Integr Physiol* **130**: 437–460.

World Health Organization (2017) Priority pathogens list for R&D of new antibiotics. URL https://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Zachowski, A. (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem J* **294**: 1–14.

Zeidler, S., and Müller, V. (2018) The role of compatible solutes in desiccation resistance of *Acinetobacter baumannii*. *Microbiology open* **8**: e740.

Zeidler, S., and Müller, V. (2019) Coping with low water activities and osmotic stress in *Acinetobacter baumannii*: significance, current status and perspectives. *Environ Microbiol* **21**: 2212–2230.

Zeidler, S., Hubloher, J., Schabacker, K., Lamosa, P., Santos, H., and Müller, V. (2017) Trehalose, a temperature- and salt-induced solute with implications in pathobiology of *Acinetobacter baumannii*. *Environ Microbiol* **19**: 5088–5099.

Zeidler, S., Hubloher, J., König, P., Ngu, N.D., Scholz, A., Averhoff, B., and Müller, V. (2018) Salt induction and activation of MtlD, the key enzyme in the synthesis of the compatible solute mannitol in Acinetobacter baumannii. *Microbiology open* **7**: e00614.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Suppl. 1 Primers used in the study