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The choline dehydrogenase BetA of *Acinetobacter baumannii*: a flavoprotein responsible for osmotic stress protection

Jennifer Breisch, Melanie Bendel and Beate Averhoff*
Department of Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Goethe-University Frankfurt am Main, Frankfurt, Germany.

Summary

***Acinetobacter baumannii* is outstanding for its ability to cope with low water activities which significantly contributes to its persistence in hospital environments. The vast majority of bacteria are able to prevent loss of cellular water by amassing osmoactive compatible solutes or their precursors into the cytoplasm. One such precursor of an osmoprotectant is choline that is taken up from the environment and oxidized to the compatible solute glycine betaine. Here, we report the identification of the osmotic stress operon *betIBA* in *A. baumannii*. This operon encodes the choline oxidation pathway important for the production of the solute glycine betaine. The salt-sensitive phenotype of a *betA* deletion strain could not be rescued by addition of choline, which is consistent with the role of BetA in choline oxidation. We found that BetA is a choline dehydrogenase but also mediates *in vitro* the oxidation of glycine betaine aldehyde to glycine betaine. BetA was found to be associated with the membrane and to contain a flavin, indicative for BetA donating electrons into the respiratory chain. The choline dehydrogenase activity was not salt dependent but was stimulated by the compatible solute glutamate.**

Introduction

Bacteria are constantly encountering changes in the physico-chemical properties of their environments (Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood *et al*., 2001). Among those is fluctuation in the availability of water and bacteria have evolved sophisticated regulatory networks to measure and to respond to changing water activities (Chowdhury *et al*., 1996; Record Jr. *et al*., 1998; Empadinhas and da Costa, 2008; Gunde-Cimerman *et al*., 2018; Bremer and Krämer, 2019). Since biological membranes are permeable to water, a reduction of the water content of the environment will automatically result in a loss of cellular water, cell shrinkage and finally death, if no countermeasures are taken (Bremer and Krämer, 2019). The vast majority of bacteria respond to low water activities by amassing compatible solutes in their cytoplasm, small, uncharged molecules that are soluble to high concentrations (Wood *et al*., 2001; Burg and Ferraris, 2008; Empadinhas and da Costa, 2008; Oren, 2008; Zeidler and Müller, 2019). Typical compatible solutes are amino acids or derivatives thereof such as glutamate, proline or ectoine (Kempf and Bremer, 1998; Roeßler and Müller, 2001). In general, synthesis of compatible solutes is energetically more costly than uptake of solutes from the environment, and thus, uptake of, for example glycine betaine, is preferred over synthesis (Oren, 1999). Indeed, the presence of glycine betaine usually represses the synthesis of solutes (Zeidler *et al*., 2017).

The opportunistic human pathogen *Acinetobacter baumannii* is known for its outstanding resistance to low water activities (Jawad *et al*., 1996; Wendt *et al*., 1997; Zeidler *et al*., 2017; Breisch *et al*., 2018; Zeidler *et al*., 2018; Zeidler and Müller, 2018; Zeidler and Müller, 2019; Breisch and Averhoff, 2020). It synthesizes glutamate and mannitol as the main compatible solutes but also trehalose in minor amounts (Zeidler *et al*., 2017; Zeidler *et al*., 2018). *A. baumannii* also has a whole set of transporters for the uptake of compatible solutes, in particular glycine betaine (BetU1, BetU2, BetU3) and its precursor choline (BetT1, BetT2, Aci01347), which are secondary active and belong to the betaine-choline-carnitine-transporter family (Breisch *et al*., 2019; Breisch and Averhoff, 2020). In general, oxidation of choline to glycine betaine can occur by four enzyme systems: a cytoplasmic type-III alcohol dehydrogenase, which catalyses the first step in choline oxidation in *Bacillus subtilis* (Boch

Received 20 May, 2021; accepted 22 August, 2021. *For correspondence. E-mail averhoff@bio.uni-frankfurt.de; Tel. + 49 69 79829509. Fax +49 69 79820306.

et al., 1996), a soluble choline oxidase, which often catalyses both steps in choline oxidation (Ikuta *et al*., 1977; Rozwadowski *et al*., 1991), a soluble choline monooxygenase, which catalyses just the first step in choline oxidation (Rathinasabapathi *et al*., 1997; Russell *et al*., 1998) and a membrane-associated choline dehydrogenase in combination with the betaine aldehyde dehydrogenase, as found in *Escherichia coli*, *Pseudomonas aeruginosa* or *Acinetobacter baylyi* (Landfald and Strøm, 1986; Russell and Scopes, 1994; Velasco-Garcia *et al*., 1999; Scholz *et al*., 2016). In *A. baylyi*, a potential choline dehydrogenase (BetA) catalyses the first step of choline oxidation and the glycine betaine aldehyde dehydrogenase (BetB) the second. The corresponding genes are organized in an operon and are under the regulation of a TetR-type transcriptional regulator (BetI) (Scholz *et al*., 2016). A *betIBA* operon was also found in *A. baumannii*, located upstream of the two transporter genes (*betT1* and *betT2*), which are essential for osmostress-dependent and -independent choline uptake (Breisch and Averhoff, 2020).

Unfortunately, not much is known about the biochemical properties of the choline dehydrogenase catalysing the first step of the formation of glycine betaine from its precursor choline. To fill this gap, we deleted the gene to unravel the phenotype of the mutant, purified the protein and studied its biochemical properties.

Results

Properties of bet *gene products*

Analyses of the genetic organization of the *betT1* and *betT2* genes, encoding the secondary active choline transporters led to the detection of divergently transcribed genes upstream of the transporter genes that encode proteins with similarity to a potential choline dehydrogenase (HMPREF0010_01460, *betA*), a betaine aldehyde dehydrogenase (HMPREF0010_01461, *betB*) as well as a potential transcriptional regulator (HMPREF0010_01462, *betI*) (Breisch and Averhoff, 2020). This *betIBA* gene cluster is also found in other bacteria such as *A. baylyi*, *Acinetobacter nosocomialis*, *E. coli*, *Halobacillus halop hilus* or *Halomonas elongata* (Fig. 1). The *betA* gene of *A. baumannii* encodes a 571 aa protein with a deduced mass of 64 kDa. BetA is very similar to BetA from *A. baylyi* (96% similarity and 90% identity), *A. nosocomialis* (98% similarity and 98% identity), *E. coli* (92% similarity and 79% identity) or *P. aeruginosa* (93% similarity and 79% identity). Secondary structure predictions revealed a Rossmann fold typical for flavine-adenine dinucleotide (FAD) or NAD(P) binding. The *betB* gene encodes a 490 aa protein with a deduced mass of 53 kDa and a predicted NAD binding site. BetB is very similar to BetB from *A. baylyi* (96% similarity and 86% identity), *A. nosocomialis* (99% similarity and 99% identity), *E. coli* (90% similarity and 75% identity) or *P. aeruginosa* (93% similarity and 78% identity). The *betI* gene encodes a 213 aa protein with a deduced mass of 25 kDa and is annotated as a TetR-type transcriptional repressor. It is also very similar to the corresponding proteins from *A. baylyi* (95% similarity and 81% identity), *A. nosocomialis* (99% similarity and 99% identity), *E. coli* (90% similarity and 75% identity) or *P. aeruginosa* (93% similarity and 78% identity).

A betA *deletion mutant is no longer able to convert choline to glycine betaine*

To get insights into the role of the choline dehydrogenase BetA of *A. baumannii* ATCC 19606 in choline oxidation, a markerless *betA* deletion mutant was generated via integration of a plasmid with a kanamycin resistance cassette and a levansucrase gene into the *betA* locus via homologous recombination followed by segregation of *betA* by counter selection in sucrose medium. The growth phenotype of the *betA* mutant was analysed by growth studies in mineral medium (MM) with 500 mM sodium chloride and in the presence or absence of choline or glycine betaine (Fig. 2). Growth of the wild type and the *betA* deletion mutant in MM in the absence of sodium chloride was comparable with growth rates of 0.65 ± 0.04 and $0.64 \pm 0.04\ h^{-1}$, respectively. Growth of both strains was similarly retarded in the presence of 500 mM sodium chloride, with growth rates of 0.21 ± 0.02 and $0.24 \pm 0.02\ h^{-1}$, respectively. Although growth of the wild type was restored by addition of choline to the medium ($0.36 \pm 0.02\ h^{-1}$), this was not observed in the *betA* mutant. Obviously, choline is no longer oxidized to the osmoprotectant glycine betaine in the *betA* mutant. The growth rate of the *betA* mutant in the presence of high salt and choline was even lower ($0.07 \pm 0.01\ h^{-1}$) than the growth rate in the presence of high salt without choline. This suggests that the accumulation of choline in the cells has an inhibitory effect. The addition of glycine betaine restored the growth of the wild type and the *betA* deletion mutant in high salinity medium. Comparable growth rates of 0.68 ± 0.02 and $0.71 \pm 0.08\ h^{-1}$, respectively were observed which clearly shows that the *betA* mutant is not impaired in using glycine betaine as compatible solute.

BetA is associated with the membrane and oxidizes choline

To produce (and later on purify) BetA, *betA* was cloned downstream of the *lac* promoter in pT7-7, a sequence

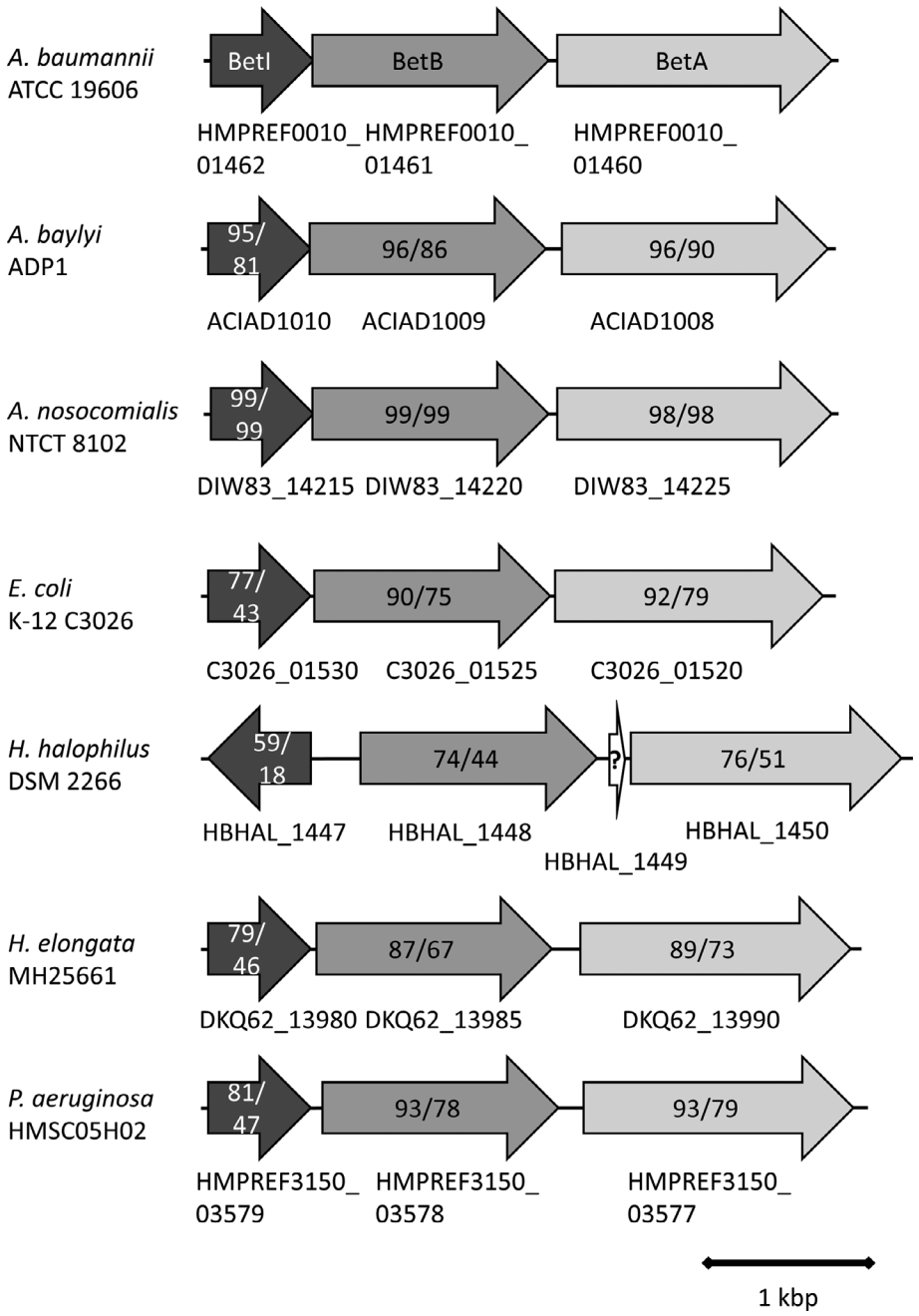

Fig. 1. Genetic organization of the *betIBA* operon in *Acinetobacter baumannii*, *Acinetobacter baylyi*, *Acinetobacter nosocomialis*, *Escherichia coli*, *Halobacillus halophilus*, *Halomonas elongata* and *Pseudomonas aeruginosa*. The genes responsible for choline oxidation are clustered in one operon in different bacteria. *BetA* encodes the choline dehydrogenase, *betB* the betaine aldehyde dehydrogenase and *betI* the TetR-type transcriptional regulator. Similarities and identities are stated within the arrows (similarity/identity).

encoding a C-terminal his_6-tag was added and gene expression in *E. coli* BL21 STAR was induced by addition of IPTG to a final concentration. Cells were harvested and a cell free extract was prepared. To measure the enzymatic activity of BetA, phenazine methosulfate (PMS) was used as primary electron acceptor. Reduced PMS is spontaneously reoxidized with concomitant consumption of oxygen which was measured with a Clark oxygen electrode. Upon addition of choline to the cell free extract, oxygen was consumed with a rate of 0.5 ± 0.1 μmol O_2 min^{-1} mg^{-1} protein. There was no oxygen consumption when choline, PMS or BetA was omitted from the assay. Cells in which BetA production had not been induced did not catalyse choline-dependent oxygen consumption, due to the missing induction of the *E. coli betIBA* operon in the overproduction strain under these growth conditions, where neither choline nor high salt concentrations were added to the culture medium (Landfald and Strøm, 1986). These studies revealed that BetA indeed oxidizes choline. The total activity in cell free

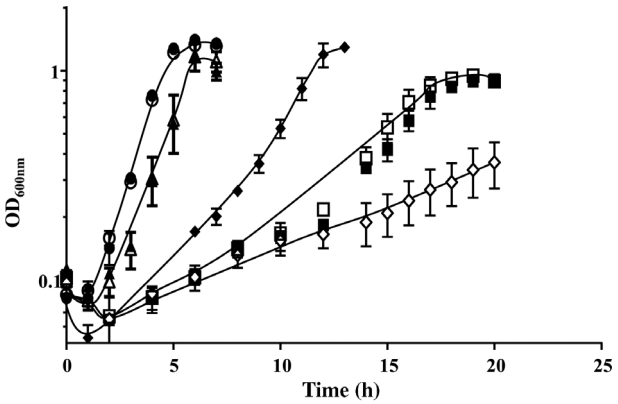

Fig. 2. Growth of *A. baumannii* ATCC 19606 wild type and *betA* mutant under osmotic stress in the presence or absence of choline or glycine betaine. *A. baumannii* ATCC 19606 wild type (filled symbols) and *betA* deletion mutant (open symbols) were grown in mineral medium (●/○), in mineral medium with 500 mM NaCl (■/□), in mineral medium with 500 mM NaCl and 1 mM glycine betaine (▲/△) and in mineral medium with 500 mM NaCl and 1 mM choline (♦/◇). Each value is the mean of ±SEM of at least three independent measurements.

extract was 291 ± 14.3 μmol O_2 min^{-1} and the specific activity was 0.5 μmol O_2 min^{-1} mg^{-1} protein. After separation of the cell free extract into membranes and supernatant, only 13% (39 ± 4.6 μmol O_2 min^{-1}) of the activity was found in the supernatant and 73% (213 ± 17.9 μmol O_2 min^{-1}) in membranes. This indicates that BetA is associated with the membrane although it does not contain obvious membrane-targeting signals or hydrophobic stretches.

Biochemical characterization of BetA

Recombinant BetA was purified by nickel-sepharose chromatography followed by separation in an SDS gel and staining with Coomassie Brilliant Blue (Fig. 3). The highly enriched BetA preparation showed two major proteins with apparent molecular masses of 66 and 36 kDa. The first value agreed well with the molecular mass deduced from the *betA* gene (M_r = 64 kDa). The proteins were cut out of the SDS gel and analysed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (Functional Genomics Center Zurich, ETH Zurich/University of Zurich, Zurich, Switzerland). These analyses confirmed that the 64 kDa protein represents BetA. The second major protein was identified as FK506-binding protein-type peptidyl-prolyl cis-trans isomerase, which catalyses the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (Takahashi *et al*., 1989). All biochemical analyses were performed with this highly enriched BetA preparation.

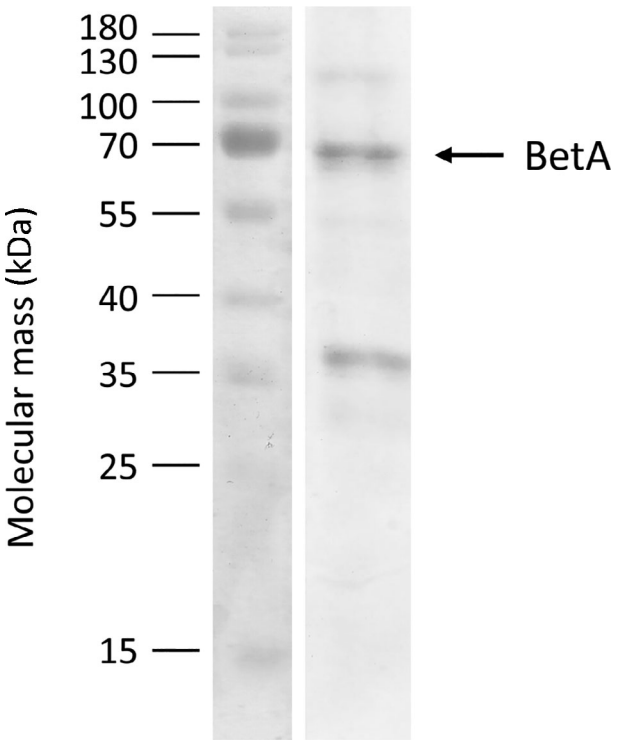

Fig. 3. Partially purified choline dehydrogenase BetA of *A. baumannii*. The enzyme was isolated via Ni-NTA (elution at 800 mM imidazole) and analysed on a 12.5% SDS gel. 20 μg of protein was applied to the gel and stained with Coomassie Brilliant Blue R-250.

We analysed the choline oxidation activity of BetA using PMS as primary electron acceptor, which is reoxidized by molecular oxygen. These analyses revealed a specific activity of BetA of 2.4 ± 0.2 μmol O_2 min^{-1} mg^{-1} protein. In contrast to the bifunctional mannitol-1-phosphate dehydrogenase MtlD, BetA activity was maximal in the absence of salts such as NaCl (Fig. 4A) or KCl (Fig. 4B). Activity declined with higher salt concentrations, only with KCl there was a little increase at 50 mM of 0.4 ± 0.3 μmol O_2 min^{-1} mg^{-1} protein. BetA-dependent choline oxidation had a clear optimum at pH 9.0 and activity was only 21.4 ± 3% at pH 7.0 (Fig. 4C) showing a clear preference for more alkaline pH values. To analyse the affinity of BetA for choline the oxygen consumption during choline oxidation was measured in dependence of different choline concentrations (Fig. 4D). These studies revealed that BetA has a low substrate affinity with a deduced K_M value of 10.3 ± 1.2 mM at a physiological pH of 7.0 and in the absence of salt.

To verify the substrate specificity of BetA oxidation of betaine aldehyde was also tested. In the presence of 5 mM betaine aldehyde and PMS an oxygen consumption rate of 0.9 ± 0.1 μmol O_2 min^{-1} mg^{-1} protein was detected. In contrast, in the presence of 5 mM choline 1.4 ± 0.3 μmol O_2 min^{-1} mg^{-1} protein was consumed.

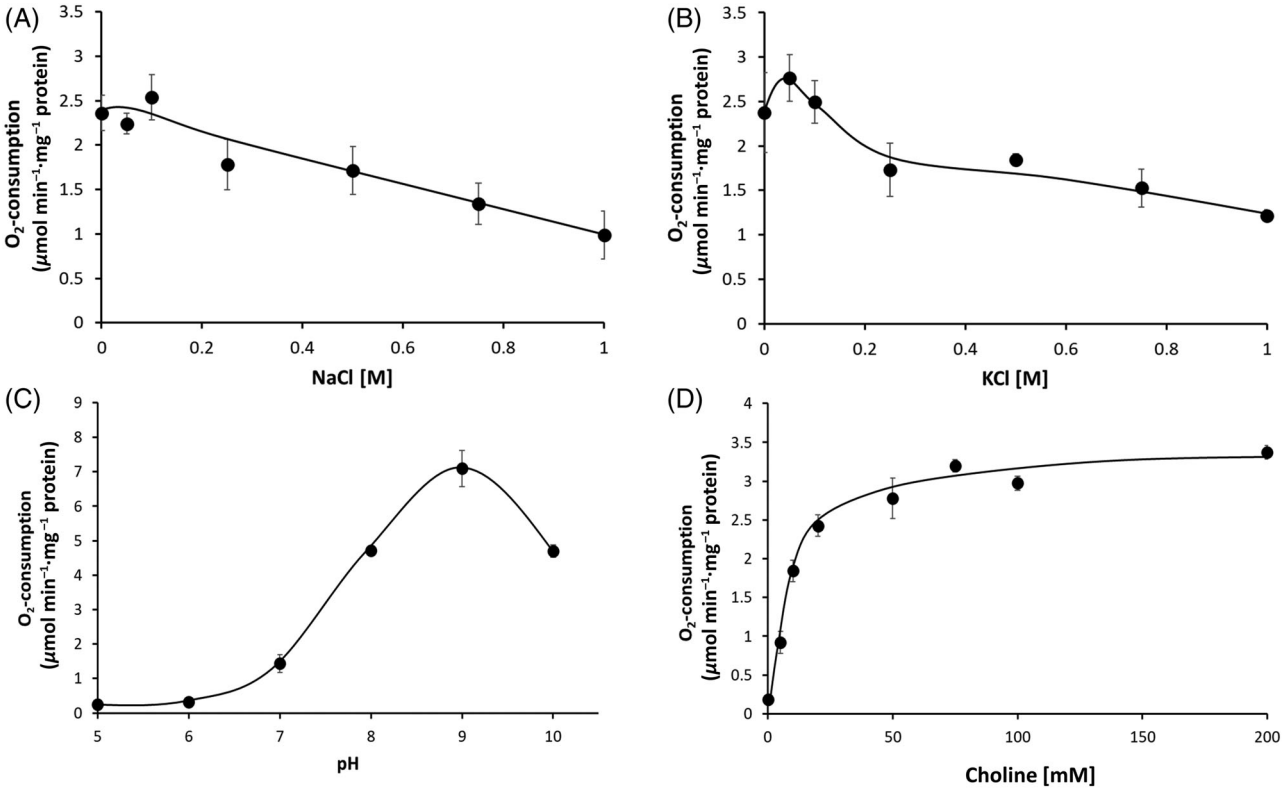

Fig. 4. Enzymatic characterization of BetA. 20 µg BetA was preincubated with 2.7 ml air-saturated buffer A or B, the reaction was started by addition of choline (20 mM) and the oxygen consumption was then measured as described in the Materials and methods section. The buffer A contained different amounts of NaCl (A) or KCl (B), initial pH was varied in buffer B between 5 and 10 (C) and the choline concentration was varied in buffer A between 0 and 200 mM (D). The activities are given in µg O_2 min^{-1} mg^{-1} protein. Each value is the mean of ±SEM of at least three independent measurements.

This suggests that BetA can catalyses both reactions, choline oxidation to betaine aldehyde and betaine aldehyde oxidation to glycine betaine.

The presence of a Rossmann fold, as well as its membrane association, prompted us to check for flavins bound to BetA. Therefore, purified BetA was precipitated by trichloroacetic acid (TCA) and the supernatant was analysed for flavins by thin-layer chromatography (TLC). As can be seen in Fig. 5, FAD but not flavine mononucleotide (FMN) was found in the enzyme. Addition of FAD (or FMN) to the buffer did not stimulate choline oxidation by BetA (data not shown), which suggests that the external electron acceptor PMS accepts the electron from the enzyme before it reaches the flavin.

BetA is stimulated in the presence of glutamate

Glutamate uptake or production is one of the first osmostress answers in bacteria (Whatmore *et al*., 1990; Saum and Müller, 2008). To address the question of whether BetA activity is stimulated by glutamate, the choline-dependent oxygen consumption of purified BetA was analysed in the presence of different glutamate concentrations (Fig. 6). These studies revealed that increasing glutamate concentrations led to increasing oxygen consumption rates with a maximum of 4.9 ± 0.1 µmol O_2 min^{-1} mg^{-1} protein in the presence of 50 mM glutamate. This leads to the conclusion that glutamate stimulates choline oxidation activity of BetA. The presence of other compatible solutes, such as trehalose or mannitol, which are synthesized *de novo* by *A*. *baumannii* had no effect on BetA activity. Choline-dependent oxygen consumption in the absence of compatible solutes and after addition of 2 mM choline was 1.3 ± 0.3 µmol O_2 min^{-1} mg^{-1} protein and in the presence of trehalose or mannitol 1.2 ± 0.2 µmol O_2 min^{-1} mg^{-1} protein or 1.3 ± 0.2 µmol O_2 min^{-1} mg^{-1} protein, respectively, whereas the oxygen consumption rate decreased in the presence of glycine betaine by 42% to 0.5 ± 0.01 µmol O_2 min^{-1} mg^{-1} protein, due to the product inhibition. This clearly suggests that the presence of *de novo* synthesized compatible solutes does not inhibit choline oxidation and that glycine betaine production is preferred during salt stress.

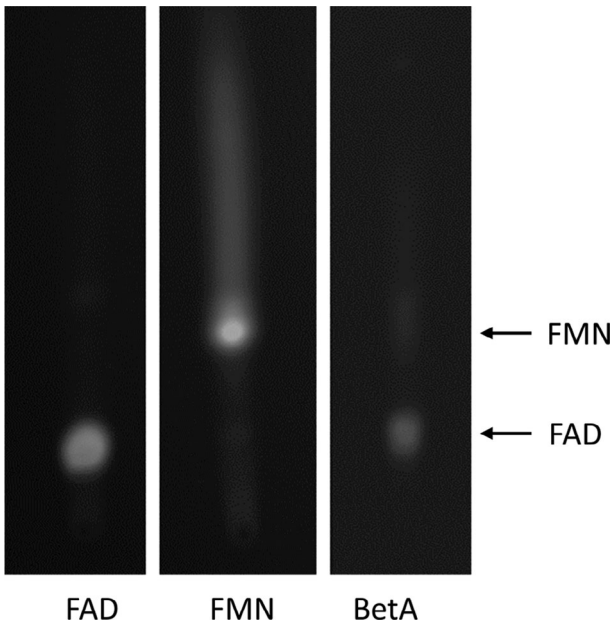

Fig. 5. BetA contains FAD. Flavin binding of BetA was analysed after TCA precipitation and TLC. 1 nmol flavin-adenine dinucleotide (FAD) and 1 nmol flavin mononucleotide (FMN) were used as standards. Flavins are detected via ultraviolet radiation. Arrows indicated the migration behaviour of FAD and FMN.

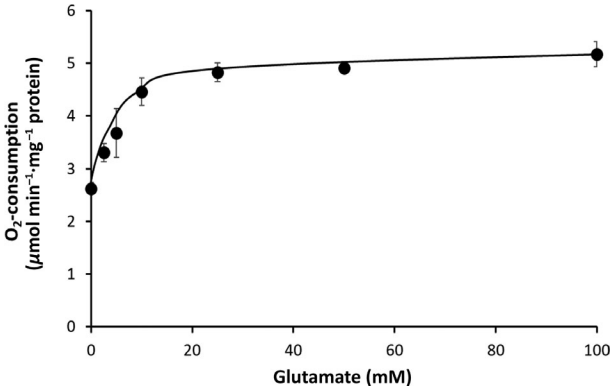

Fig. 6. Stimulation of choline oxidation by glutamate. 20 μg BetA was preincubated with 2.7 ml air-saturated buffer A containing 0–100 mM glutamate, the reaction was started by addition of choline (20 mM) and the oxygen consumption was then measured as described in the Materials and methods section. The activities are given in μg O_2 min^{-1} mg^{-1} protein. Each value is the mean of ±SEM of at least three independent measurements.

Discussion

In this study, we have addressed the role of *betA* in *A. baumannii*. A *betA* deletion mutant was significantly impaired in growth with high salt and growth of the mutant could not be restored by the addition of choline as expected if choline is oxidized to the compatible solute glycine betaine. Interestingly, the *betA* mutant exhibited a decreased growth rate in the presence of high salt and choline in comparison to the growth rate with high salt in the absence of choline. This indicates that the accumulation of the positively charged choline in the *betA* deletion mutant under high salt conditions, which we have confirmed in previous studies with a *A. baylyi betA* mutant (Scholz *et al*., 2016), has a growth inhibitory effect. This has already been reported also for a *B. subtilis* mutant lacking the glycine betaine-biosynthetic enzymes (Boch *et al*., 1996).

So far, a biochemical characterization of choline dehydrogenases has been severely impeded by the instability and problems to obtain highly purified enzymes (Gadda, 2020). Up to now, only three bacterial choline dehydrogenases have been purified such as the enzymes from *H. elongata*, *E. coli* and a *Pseudomonas* strain (Russell and Scopes, 1994; Gadda and McAllister-Wilkins, 2003; Rajan *et al*., 2010). Choline oxidation studies with a highly purified BetA preparation of *A. baumannii* provided clear evidence that the enzyme uses not only choline but also glycine betaine aldehyde as substrate in the presence of PMS as primary electron acceptor which is reoxidized by molecular oxygen. However, it has to be noted that the BetA-mediated glycine betaine aldehyde oxidation activity was significantly lower than the choline oxidation activity. Since it is known that betaine aldehyde is a toxic intermediate we suggest that BetB is important to prevent the intracellular accumulation of glycine betaine aldehyde (Boch *et al*., 1996). In the absence of primary electron acceptors, the choline dehydrogenase could not utilize molecular oxygen indicating that BetA could not act as an oxidase. This differs from the choline dehydrogenase of *H. elongata* which was found to act as oxidase or choline dehydrogenase depending on the availability of alternative primary electron acceptors (Gadda and McAllister-Wilkins, 2003).

The finding that BetA uses choline and glycine betaine aldehyde as substrate has also been reported for the choline dehydrogenase of *H. elongata* and *E. coli* and the choline oxidase of *Arthrobacter globiformis* (Ikuta *et al*., 1977; Styrvold *et al*., 1986; Gadda and McAllister-Wilkins, 2003). The K_M value for choline was 10.3 mM and in the range with the K_M value of other choline dehydrogenases, for example from *H. elongata* ($K_M = 11.6$ mM), *P. aeruginosa* ($K_M = 1.7$ mM) and *E. coli* ($K_M = 1.5$ mM) (Nagasawa *et al*., 1976; Landfald and Strøm, 1986; Gadda and McAllister-Wilkins, 2003). The pH optimum of 9.0 of BetA from *A. baumannii* is similar with the pH optimum of the choline dehydrogenase of *P. aeruginosa* and the choline oxidase of *A. globiformis* (Nagasawa *et al*., 1976; Ikuta *et al*., 1977).

We found that BetA activity was not stimulated but rather inhibited by high NaCl or KCl concentrations. This differs from the stimulatory effect of NaCl and KCl on other enzymes important for osmostress protection, such

as the mannitol-1-phosphate dehydrogenase/phosphat ase of *A. baumannii* which shows the highest activities in the presence of 700 mM NaCl and is completely inactive in the absence of salt (Zeidler *et al*., 2018). Also, in *E. coli* the trehalose-phosphate synthase and the glutamate dehydrogenase are stimulated nearly 10 times by high K^+ concentrations (Measures, 1975; Giaever *et al*., 1988). However, glutamate stimulated choline oxidation. This is consistent with the fact that in the temporal order of solute accumulation, glutamate is the first. Synthesis of glutamate follows the rapid uptake of K^+ after osmotic upshock, to ensure electroneutrality. Then, K^+ and glutamate pools decrease, whereas glycine betaine increases, as its already shown for the *de novo* synthesis of solutes in *A. baumannii* ATCC 19606 (König *et al*., 2020). It is tempting to speculate that glutamate is involved in this 'solute switch', on a transcriptional but also activity level (Csonka and Epstein, 1996; Gralla and Vargas, 2006).

The presence of a Rossmann fold and moreover the typical glycine box G-X-G-X-X-G at the N-terminus of BetA suggests that the enzyme might use FAD as cofactor for choline oxidation. This glycine box (GAGSAG) is also present in the enzyme of *H. elongata* but no FAD cofactor was found in the purified choline dehydrogenase (Gadda and McAllister-Wilkins, 2003). Moreover, the enzyme from a *Pseudomonas* strain, which also contains the G-X-G-X-X-G glycine box, did not require FAD but pyrroloquinoline quinone for choline oxidation (Russell and Scopes, 1994). So, the presence of a conserved FAD-binding box does not allow the conclusion that indeed FAD is used as cofactor. To our knowledge, this is the first report on the detection of a non-covalently bound FAD in a choline dehydrogenase, although it has to be noted that FAD has been identified already in other choline oxidizing enzymes, such as choline oxidases. These enzymes contain a covalently bound FAD and use molecular oxygen as primary electron acceptor (Ohta-Fukuyama *et al*., 1980; Gadda, 2003; Gadda, 2020). The detection of FAD bound to the choline dehydrogenase of *A. baumannii* together with our recent finding that choline oxidation leads to increased ATP levels suggest that the electrons derived from choline oxidation are funnelled *via* FAD into the respiratory chain thereby leading to an energetic benefit in choline-rich host environments (Breisch and Averhoff, 2020).

Materials and methods

Bacterial strains and culture conditions

Escherichia coli BL21 STAR was grown in LB medium (Bertani, 1951) at 37°C in the presence of 100 μg ml^{-1} ampicillin. *Acinetobacter baumannii* strains were grown at 37°C in LB medium (Bertani, 1951) or in MM that consists of 50 mM phosphate buffer, pH 6.8 and different salts [per litre: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 (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* strains under hyperosmotic conditions was analysed in MM with 500 mM NaCl and in the presence or absence of 1 mM choline or 1 mM glycine betaine. 50 μg ml^{-1} kanamycin was added when appropriate. The growth experiments were repeated three times, and each value is the mean of ±SEM. Growth curves were fitted manually.

Markerless mutagenesis of A. baumannii *ATCC 19606*

To generate the markerless mutant *ΔbetA*, 1500 bp upstream and 1500 bp downstream of *betA* 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 electrocompetent *A. baumannii* wild type cells. 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_{MilliQ}, 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^{-1} 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 additional 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).

Cloning, expression and purification of BetA

To express *betA* in *E. coli* BL21 STAR it was cloned with a sequence encoding a C-terminal his_6-tag into the multiple cloning site of the expression vector pT7-7 using EcoRI and PstI. Primer pairs used for amplification of the gene and the vector are listed in Supporting Information Table S1.

Single colonies of *E. coli* BL21 STAR harbouring plasmid pT7-7_*betA-his* were used to inoculate 50 ml LB medium containing 100 μg ml^{-1} ampicillin in a 250 ml baffled flask. After 16–18 h of incubation at 37°C and

150 rpm, the culture was used to inoculate 2 L of 2× YT medium (5 g L^{-1} NaCl, 16 g L^{-1} tryptone, 10 g L^{-1} yeast extract) containing 100 μg ml^{-1} ampicillin to an OD_{600} of 0.1. After 48 h incubation under the same conditions as described above, gene expression was induced for 1 h by addition of IPTG to a final concentration of 1 mM. Afterwards, cells were harvested by centrifugation at 8000*g* for 7 min at 4°C, washed in 50 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 6.8) and stored at −20°C.

To purify BetA, frozen cells were thawed on ice and resuspended in 15 ml lysis buffer containing DNase and 0.5 mM PMSF. Cells were disrupted via French Press (three times, 1000 psi) and cell debris was removed via centrifugation (14 000*g*, 30 min, 4°C). Cell-free lysate was incubated with 2 ml of Ni-NTA material for 30 min and shaken at 4°C and then filled into a column. The column was washed two times with 50 ml washing buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 6.8) and 10 ml washing buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 70 mM imidazole, pH 6.8). Afterwards, BetA was eluted with 10 ml elution buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, pH 6.8) and 10 ml elution buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 800 mM imidazole, pH 6.8). The protein concentration was determined by Bradford (1976). Protein composition of the different fractions was analysed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (1970) stained with Coomassie (0.5 g L^{-1} Serva Blue R-250, 100 ml L^{-1} methanol, 100 ml L^{-1} glacial acetic acid).

Coupled enzymatic assay to study BetA-mediated choline oxidation activity of BetA

The choline dehydrogenase activity of BetA was studied in a coupled enzymatic assay, measuring oxygen consumption during choline oxidation, using PMS as primary electron acceptor, which is enzymatically reduced and spontaneously reoxidized by molecular oxygen. One unit of enzymatic activity corresponds to the conversion of 1 μmol O_2 min^{-1}. For measuring dehydrogenase activity, 20 μg BetA was preincubated in 2.7 ml air-saturated buffer A (50 mM NaH_2PO_4, 10 mM imidazole, 1 mM PMS, pH 7.0) for 3 min, stirring at 37°C. The reaction was started by addition of 20 mM choline and the oxygen consumption was monitored for 3 min in an oxygen electrode (Digital Model 10, Rank Brothers). The pH optimum was determined using a buffer mixture of 50 mM CHES, 50 mM TES and 50 mM Tris with 1 mM PMS (buffer B) instead of the NaH_2PO_4 buffer. Substrate dependence and K_M values were analysed using different concentrations of choline or betaine aldehyde. Stimulation of BetA via glutamate was studied by preincubation of BetA with different concentrations of glutamate for 3 min.

Analysis of flavins via thin-layer chromatography

To determine flavins 2 nmol of protein was treated with 0.2 volumes of 1 M TCA and centrifuged for 5 min at 17 000*g*. The supernatant was spotted on a Silica gel TLC plate (ALUGRAM®Xtra SILGUR, layer thickness 0.2 mm, Machery-Nagel) and flavins were eluted with 60% n-butanol, 15% acetic acid and 25% H_2O as mobile phase. Afterwards, flavins were detected with ultraviolet radiation. 1 nmol FAD and 1 nmol FMN were used as standards.

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Supporting Information

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Appendix S1: Supporting Information