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Glycine betaine metabolism in the acetogenic bacterium *Acetobacterium woodii*

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Summary

The quarternary, trimethylated amine glycine betaine (GB) is widespread in nature but its fate under anoxic conditions remains elusive. It can be used by some acetogenic bacteria as carbon and energy source but the pathway of GB metabolism has not been elucidated. We have identified a gene cluster involved in GB metabolism and studied acetogenesis from GB in the model acetogen *Acetobacterium woodii.* GB is taken up by a secondary active, Na^+ coupled transporter of the betaine-choline-carnitine (BCC) family. GB is demethylated to dimethylglycine, the end product of the reaction, by a methyltransferase system. Further conversion of the methyl group requires CO_2 as well as Na^+ indicating that GB metabolism involves the Wood-Ljungdahl pathway. These studies culminate in a model for the path of carbon and electrons during acetogenesis from GB and a model for the bioenergetics of acetogenesis from GB.

Introduction

Quaternary amines are abundant in nature and serve various functions in bacteria, archaea and eukaryotes. Carnitine is widespread in eukaryotes where it is essential for fatty acid metabolism (Longo *et al*., 2016). Glycine betaine (GB, *N,N,N*-trimethylglycine) is probably the most widespread quarternary amine found in living cells (Rhodes and Hanson, 1993; Galinski, 1995; Ventosa *et al*., 1998; Roeßler and Müller, 2001). It is produced by many plants and algae in response to increasing osmolarities (Rhodes and Hanson, 1993; Sakamoto and Murata, 2002). Accumulation of GB counterbalances external osmolarity and thus prevents loss of cellular water and restores turgor (Kempf and Bremer, 1998; Roeßler and Müller, 2001). A few bacteria can synthesize GB *de novo* by methylation of glycine (Galinski and Trüper, 1994; Roeßler and Müller, 2001) or in an oxidative two-step process from its precursor choline (Lamark *et al*., 1991; Boch *et al*., 1996; Burkhardt *et al*., 2009), another quaternary amine that stems from degradation of, for example, phosphatidylcholine. Many, if not all, bacteria can take up GB from the environment under osmostress conditions (Kempf and Bremer, 1998; Pflüger and Müller, 2004). Synthesis as well as activity of known GB transporters is salt activated (Kempf and Bremer, 1998; Ziegler *et al*., 2010).

Because of its spread, it is not surprising that GB serves as carbon and energy source for many microbes. Under oxic conditions, it is oxidatively demethylated by monooxygenases to yield dimethylglycine and/or sarcosine (monomethylglycine) and glycine (Wargo, 2013). Under anoxic conditions two routes exist: one is a reduction of GB to trimethylamine and acetyl phosphate catalysed by GB reductase (Möller *et al*., 1984; Hormann and Andreesen, 1989; Meyer *et al*., 1995), the other route is the demethylation to DMG, mediated by methyltransferases (Müller *et al*., 1981; Heijthuijsen and Hansen, 1989; Watkins *et al*., 2014) and a further metabolism of the methyl group.

There are only a few physiological groups of anaerobic microorganisms that can grow on GB. These are the methanogenic archaea (Watkins *et al*., 2014), the sulphate-reducing (Heijthuijsen and Hansen, 1989) and the acetogenic bacteria (Müller *et al*., 1981; Eichler and Schink, 1984). They have in common the reductive acetyl-CoA pathway or Wood-Ljungdahl pathway (WLP) that is designed to metabolize C_1 compounds such as methyl groups (Ljungdahl, 1994; Ragsdale, 2008; Fuchs, 2011; Schuchmann and Müller, 2014). Acetogenic bacteria are strict anaerobes that constitute an important link in the anaerobic food web (Drake, 1994; Schink, 1997). Acetogens convert GB to acetate thus providing a link to aceticlastic methanogens, the last limb in anaerobic food webs. Acetogens can metabolize GB in different ways. One is by reduction to acetate and trimethylamine, if another electron donor such as formate is available (Andreesen, 1994). A

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betaine reductase is also present in *Sporomusa ovata*, but the trimethylamine produced is further demethylated; the methyl groups are oxidized to CO_2 giving the electrons for betaine reduction (Möller *et al*., 1984; Visser *et al*., 2016). The other way is by demethylation of GB and further metabolizing the methyl group. This was discovered already in 1981 in *Eubacterium limosum* (Müller *et al*., 1981) and later in 1984 in *S. ovata* (Möller *et al*., 1984). But since then only a few studies have addressed the biochemistry of acetogenesis from GB by demethylation. Inspection of the genome sequence of the model acetogen *Acetobacterium woodii* revealed a number of candidate gene clusters for GB demethylation. Herein, we describe the identification of the genes involved in transport and utilization of GB as well as the function of the encoded proteins.

Results

Growth of A. woodii *on GB*

Cells of *A. woodii* transferred from a fructose-grown preculture (10 mM fructose) into the same minimal medium containing 50 mM GB showed a lag phase of about 60 h before growth started, indicating the induction of genes required for GB metabolism. GB-adapted cultures in complex medium grew with a doubling time of 7.3 h to a final optical density of 1.05 (Supporting Information Fig. S1). Final optical densities were dependent on the GB concentration with a maximum reached at around 50 mM GB (Fig. 1). Interestingly, higher GB concentrations led to more acetate production but not to an increase in OD (Fig. 1), indicating an uncoupling of growth and acetogenesis at high substrate concentrations. Dimethylglycine (DMG), sarcosine or trimethylamine did not support growth of *A. woodii* (Supporting Information Fig. S1).

Identification of genes involved in GB metabolism

Demethylation of a methyl group containing substrate is brought about by a substrate-specific methyltransferase I (MTI) that abstracts the methyl group and transfers it to a corrinoid protein (CoP). From there the methyl group is transferred to an acceptor such as coenzyme M (in methanogens) or tetrahydrofolic acid (in acetogens) by a methyltransferase II (MTII) (van der Meijden *et al*., 1983a, 1983b; Sauer *et al*., 1997; Sauer and Thauer, 1997). Since methyl-corrinoid is a common intermediate in methyl group metabolism, genomes may encode less MTII's than MTI's. The genome of *A. woodii* encodes ~23 different MTI's, six of those potentially code for N-methyl methyltransferases (Poehlein *et al*., 2012). Of those, only four are associated with genes encoding potential secondary active GB transporters of the BCC family, indicating their role in methyl group metabolism. To determine whether any of those four MTI genes is upregulated during growth on GB, cells were grown on GB, methanol or fructose, RNA was isolated at early exponential growth phase, transcribed into cDNA and hybridized against a DNA fragment derived by PCR from each of the four MTI's. As can be seen in Fig. 2, *mttB3* (Awo_c05430), *mttB4* (Awo_c05440) and *mttB5* (Awo_c05470) were not expressed during growth on fructose, methanol or GB. Only *mttB10* (Awo_c07540) was expressed during growth on GB, but not during growth on fructose or methanol, indicating that it is involved in GB metabolism.

Properties of putative glycine betaine utilization genes and deduced proteins

The gene *mttB10* is apparently part of a bigger gene cluster (Fig. 3A). The first gene in the cluster, *mttA2* (Awo_c07520), is 921 bp long and codes for a protein of 33.7 kDa. MttA2 shares 67% identity with methylcorrinoid:tetrahydrofolate methyltransferase MtgA from *Desulfitobacterium hafniense.* MtgA catalyses the transfer of a methyl group from methylcobalamin to THF (Ticak *et al*., 2014). Therefore, a similar function of MttA2 is likely. The gene *opuD2* (Awo_c07530) is located 42 bp downstream of *mttA2* and comprises 1563 bp. The predicted molecular mass of the deduced protein is 56.5 kDa. OpuD2 shows identities of 43% and 40% to OpuD from *Bacillus subtilis* (Kappes *et al*., 1996) and BetP from *Corynebacterium glutamicum* (Peter *et al*., 1996), respectively. OpuD, as well as BetP, belong to the BCC family of transporters and have been identified as Na^+-dependent, secondary active GB symporters which catalyse GB import into the cells (Kappes *et al*., 1996; Ressl *et al*., 2009; Ziegler *et al*., 2010). The following gene in the cluster, *mttB10* (Awo_c07540), is located 35 bp downstream of *opuD2.* It is 1431 bp long and encodes a protein of

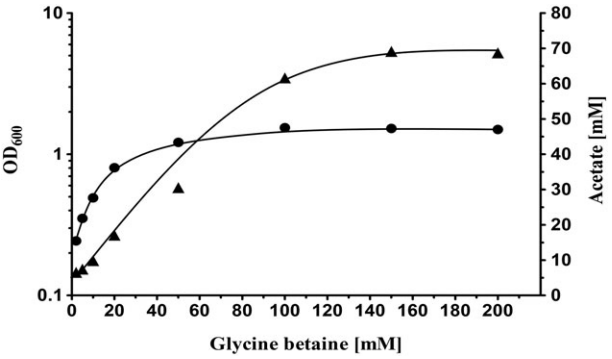

Fig. 1. Acetate formation from GB by *A. woodii.* The cultures were grown on different concentrations of GB. After 60 h final optical densities at 600 nm were measured (●) and corresponding acetate concentrations were determined by gas chromatography (▲).

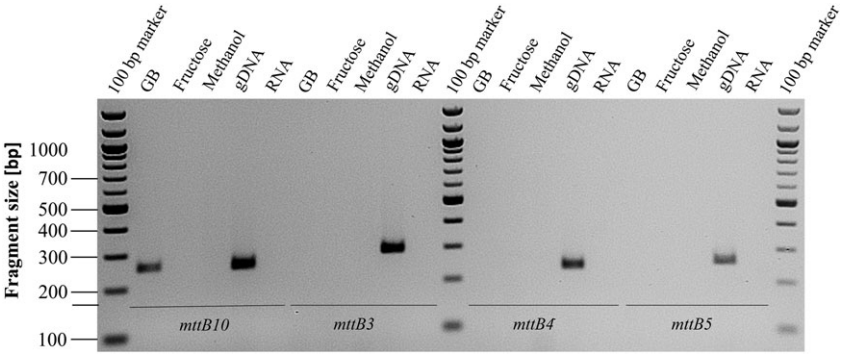

Fig. 2. Expression levels of the putative MTI genes of *A. woodii* during growth on fructose, GB or methanol. *A. woodii* was grown on different substrates as indicated. RNA was isolated and transcribed into cDNA. The transcript abundance of the four putative MTI genes was analysed as described in experimental procedures. gDNA, genomic DNA.

52.17 kDa. The deduced protein shares 68% identity with the MTI MtgB from *D. hafniense* which catalyses the demethylation of GB to DMG and a subsequent transfer of the methyl group to cobalamin (Ticak *et al*., 2014). Indicated by the high sequence similarity MttB10 is possibly a glycine betaine:corrinoid MTI. 28 bp downstream of *mttB10* follows *mttC6* (Awo_c07550) with a length of 651 bp. The encoded protein MttC6 has a predicted molecular mass of 22.9 kDa, contains a corrinoid binding domain and exhibits an identity of 45% to MtbC from *Methanosarcina barkeri* which is involved in methyl group transfer from dimethylamine to coenzyme M during methanogenesis (Ferguson *et al*., 2000), thus indicating that it encodes the corrinoid protein of the methyltransferase system. With a distance of 93 bp the gene *opuD3* (Awo_c07560) is located downstream of *mttC6.* It has a length of 1467 bp and encodes a protein of 54 kDa. The deduced protein shares sequence identities of 52% and 43% with the above-mentioned BCCTs OpuD and BetP, respectively. OpuD2 and OpuD3 are 42.2% identical and

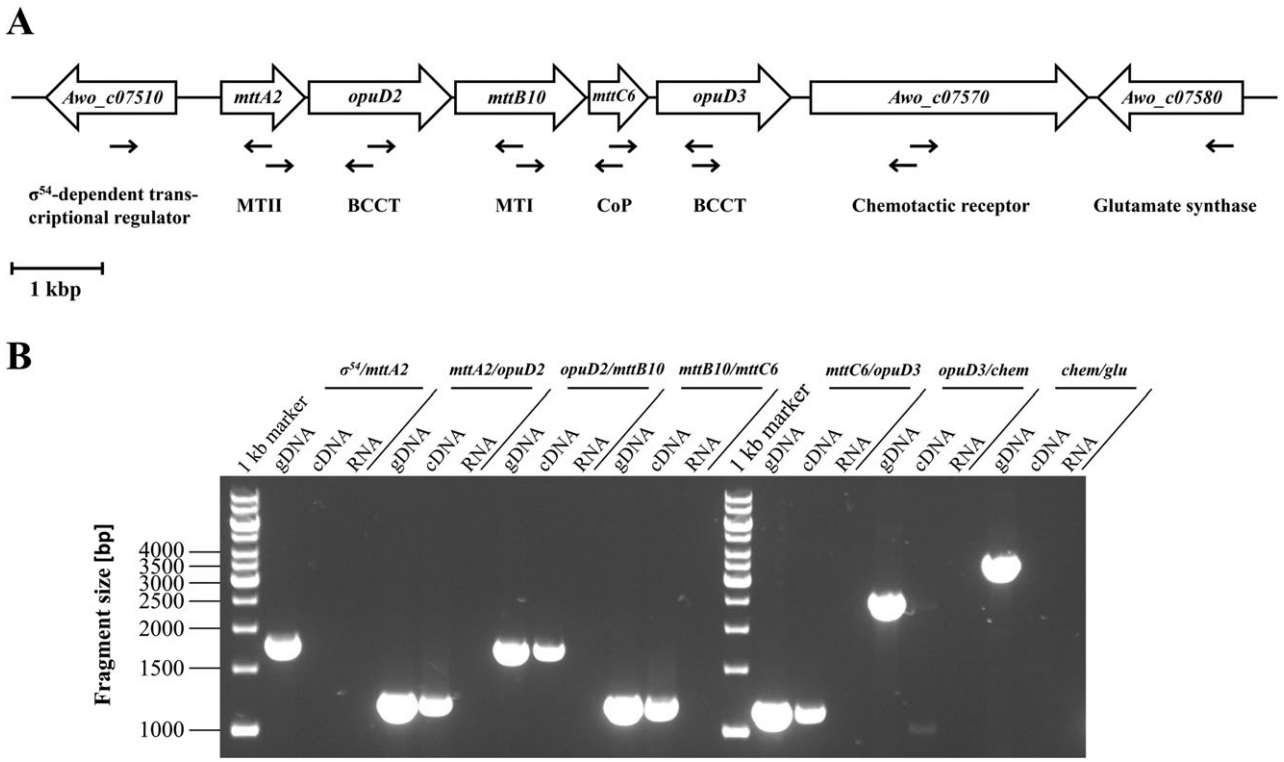

Fig. 3. Arrangement and expression analysis of the GB utilization genes of *A. woodii.* A. Genomic organization of the putative GB utilization genes of *A. woodii.* Sequences of depicted genes are available by accession numbers Awo_c07510–07580 at the database of National Center for Biotechnology Information (NCBI). Primer pairs for analysis of transcriptional organization, which bridge the intergenic regions are indicated by arrows. Putative gene products are indicated below the genes. BCCT, putative betaine/choline/carnitine transporter; CoP, putative corrinoid protein; MTII, putative methyltransferase II; MTI, putative methyltransferase I. B. The putative GB utilization genes constitute an operon. *A. woodii* was grown on GB (50 mM). RNA was isolated and transcribed into cDNA. The transcriptional organization of the putative GB utilization cluster was analysed by PCR with primers bridging the intergenic regions as described in experimental procedures.

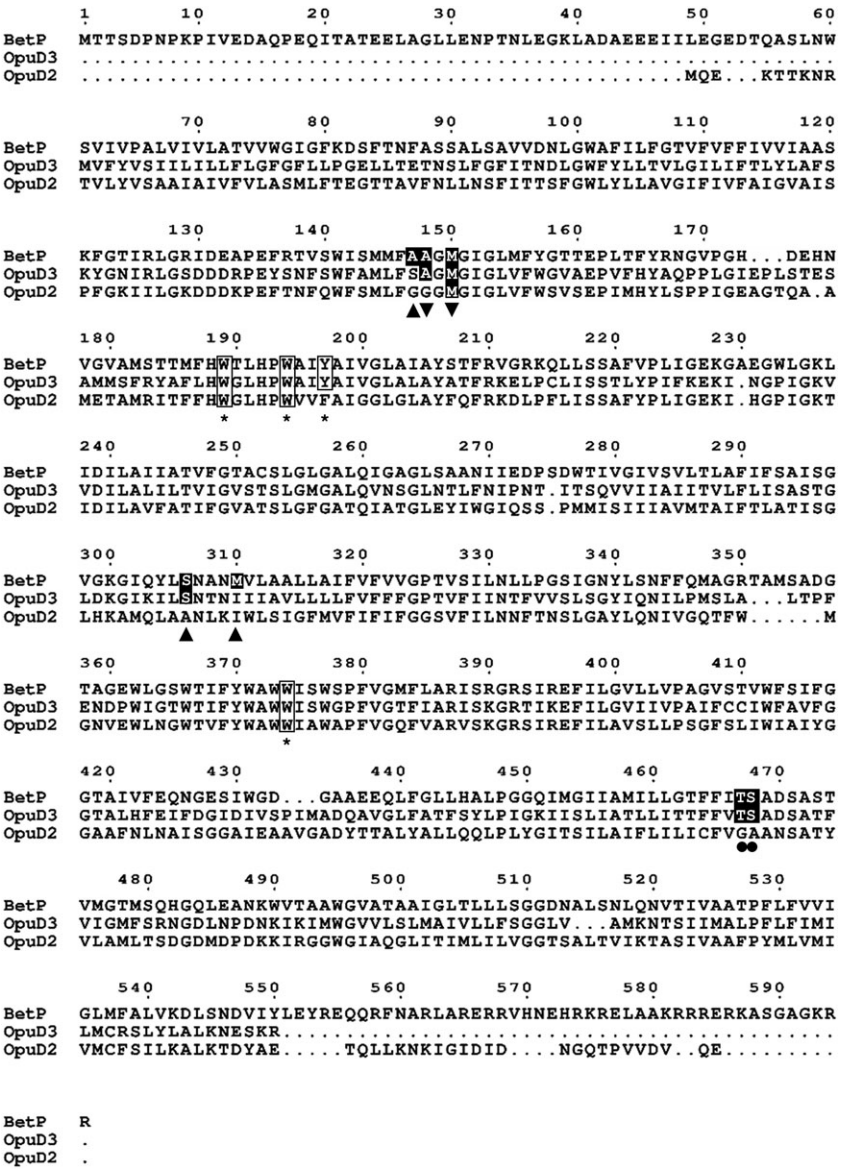

Fig. 4. Amino acid sequence alignment of Na^+/GB symporter BetP from *C. glutamicum* with putative BCC transporters OpuD3 and OpuD2 from *A. woodii*. Sequences were aligned using Clustal Omega multiple sequence alignment and visualized by ESPript 3.0 (http://espript.ibcp.fr). The GB binding aromatic cage is indicated by * and black boxes. The potential Na^+ binding sites are indicated by (▼) ('Na1'), (▲) ('Na2') and filled black boxes. Amino acids participating in binding of both sodium ions are indicated by (●).

75.7% similar to each other (Fig. 4). Membrane topology predictions of OpuD2 and OpuD3 prognosticated 12 trans-membrane (TM) spanning helices for both putative transporters as well as N- and C-terminal extensions looming into the cytoplasm. Both features are characteristic of many BCCTs (Ziegler *et al*., 2010). Furthermore, the predicted cytosolic C-terminus is relatively short for both proteins. Longer C-termini have been associated with positive regulatory functions in context of osmotic stress (Peter *et al*., 1998; Schiller *et al*., 2004; Ziegler *et al*., 2010). OpuD2 and OpuD3 both have the conserved amino acid motif GMGIG, which to date has only been found in GB-specific BCCTs (Ziegler *et al*., 2010), moreover, the 'aromatic cage' (W189, W194, Y197 and W374) involved in GB binding is also highly conserved in OpuD3 whereas OpuD2 lacks the conserved tyrosin Y197 (Fig. 4) (Ressl *et al*., 2009; Schulze *et al*., 2010; Tang *et al*., 2010). While W194 is known to be conserved in all transporters of the BCCT family, W189, Y197 and W374 are only conserved in transporters of quarternary ammonium substrates (Ressl *et al*., 2009). BetP from *C. glutamicum* translocates GB together with two Na^+ and has two Na^+ binding sites. While sequence analyses revealed that Na^+ binding site 'Na1' found in BetP (M150, A148, T467, S468) is highly conserved in OpuD3, OpuD2, however, does contain only one of these amino acids. Sodium binding site 'Na2' is also conserved in OpuD3 (3 out of 5 amino acids), but was not found in OpuD2 (Fig. 4), suggesting that it is not Na^+ coupled. In summary these findings as well as the sequence similarities of both proteins to the known GB-

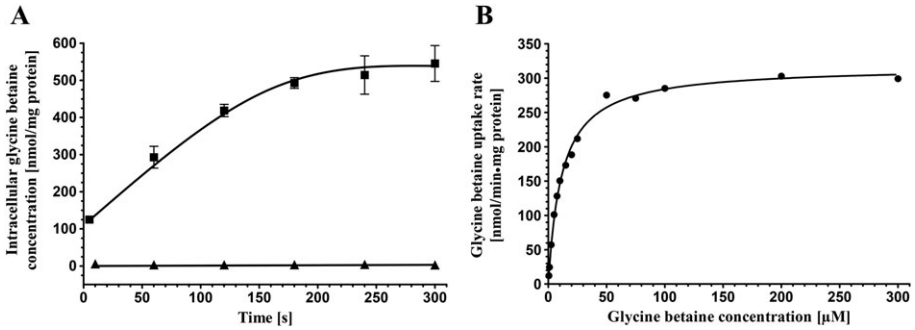

Fig. 5. OpuD3 is a GB transporter. [^{14}C]glycine betaine uptake of OpuD3 from *A. woodii* (A) was determined in *E. coli* MKH13 producing OpuD3 (■) or in cells containing the cloning vector pBAD/His (▲). Measurements were performed in 25 mM KPi buffer containing 100 mM NaCl at 37°C. Uptake was started by adding 1 mM carrier-free [^{14}C]glycine betaine (0.1 µCi/ml). Each value is the mean ± S.E.M. of three independent measurements. The kinetic constants (B) were derived by Michaelis–Menten curve fitting of uptake rates versus substrate concentration with GraphPad Prism version 4.03 for Windows (GraphPad Software, USA).

specific BCCTs BetP and OpuD indicate that OpuD2 and OpuD3 may also be GB-specific BCCTs. One or both putative transporters could be involved in uptake of GB in *A. woodii.* 214 bp downstream of *opuD3* is the gene Awo_c07570 with a length of 3042 bp. The encoded protein has a molecular mass of 110.1 kDa and shares 38% similarity with Tar from *Escherichia coli.* Tar is a chemotactical receptor protein which mediates taxis to attractants (Silverman and Simon, 1977; Falke *et al*., 1997). 91 bp downstream of Awo_c07570 is a putative glutamate synthase gene (Awo_c07580) in inverted orientation. 470 bp upstream of *mttA2* is Awo_c07510 in inverted orientation with a length of 1425 bp. It is annotated as σ^{54}-dependent DNA-binding response regulator. The gene encodes a protein of 54.4 kDa containing a σ^{54}-interaction domain. The deduced protein has an identity of 30% to the σ^{54}-dependent transcriptional activator RocR from *B. subtilis* (Calogero *et al*., 1994; Gardan *et al*., 1997). Promoter prediction revealed a putative σ^{54}-dependent promoter region 159 bp upstream of the first gene of the cluster, *mttA2*, containing conserved −12/−24 sequences (5'-AAAATAACGAAGATTCTGAATAAATTGAGTTTGGCATGATAATTGCGTTATCTAATATCACAAGTTGAAATGGCCTTTCACA-3′) (Barrios *et al*., 1999; Lin *et al*., 2014). The gene product of Awo_c07510, therefore, may control the transcriptional activation of the gene cluster. In addition a rho-independent transcriptional terminator sequence was predicted 25 bp downstream of Awo_c07570 (Naville *et al*., 2011).

The putative glycine betaine utilization genes are transcribed in a polycistronic message

To analyse whether the putative GB utilization genes are organized in an operon, mRNA was isolated from *A. woodii* grown on GB and transcribed into cDNA. This cDNA was then used as a template in a PCR reaction using primers (Supporting Information Table S1) that bridge the intergenic regions between Awo_c07510/*mttA2*, *mttA2*/*opuD2*, *opuD2*/*mttB10*, *mttB10*/*mttC6*, *mttC6*/*opuD3*, *opuD3*/Awo_c07570, Awo_c07570/Awo_c07580 (Fig. 3A). Only a cDNA originating from one polycistronic mRNA is able to give products with the expected fragment sizes of 1767, 1189, 1723, 1180, 1140, 2479 and 3570 bp respectively. As can be seen from Fig. 3B, expected PCR products were obtained for the intergenic regions of *mttA2*/*opuD2*, *opuD2*/*mttB10*, *mttB10*/*mttC6* and *mttC6*/*opuD3* but not for the others. No PCR products were generated when the corresponding mRNA was used as template. This result demonstrates that *mttA2*, *opuD2*, *mttB10*, *mttC6* and *opuD3* constitute an operon. The genetic data imply that GB is transported into the cell by the BCC transporter OpuD2 and/or OpuD3 and then demethylated by MTI, CoP and MTII. This hypothesis was addressed in the following.

OpuD3 is a GB transporter

The gene *opuD3* was cloned into pBAD/His thereby introducing a His-tag encoding sequence at its 5′-terminal end. The construct was verified by DNA sequencing and transformed into *E. coli* MKH13, a strain in which all GB transporter genes have been deleted (Haardt *et al*., 1995). OpuD3 was produced and inserted into the cytoplasmic membrane, as evident from immunological analyses using an antibody against the His-Tag. Cells of *E. coli* MKH13-OpuD3 were cultivated in LB and resting cells were prepared. When these cells were suspended in buffer containing 100 mM NaCl, radioactively labelled [^{14}C]GB given to the medium was taken up by the cells. In contrast, *E. coli* cells containing the cloning vector did not take up GB (Fig. 5A). This is clear evidence that OpuD3 transports GB. Competition experiments were performed with a 50-fold molar excess of the competitor and intracellular GB concentrations after 5 min of incubation were compared. Glycine and sarcosine inhibited GB transport by 36% and 31% whereas DMG had a more

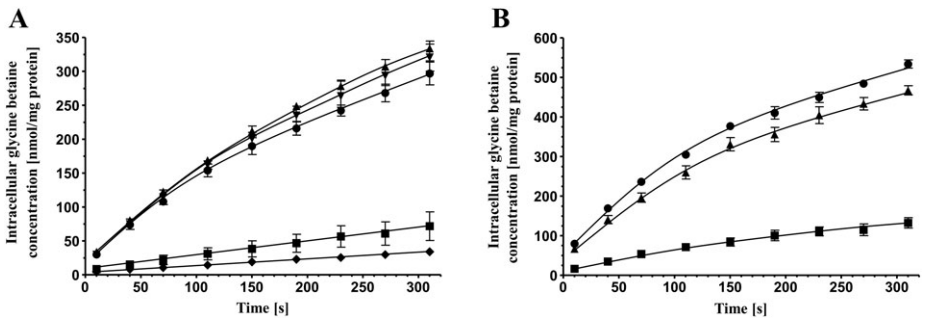

Fig. 6. GB transport is strictly Na^+ dependent and inhibited by the protonophore TCS. [^{14}C]glycine betaine uptake of OpuD3 from *A. woodii* was determined in *E. coli* MKH13 producing OpuD3. Measurements for analysis of Na^+-dependence (A) were performed in 25 mM sodium-free Tris buffer in the presence of either 100 mM NaCl (●), 100 mM NaCl and 30 μM ETH2120 (▼), 100 mM NaCl and 0.3% [v/v] DMSO (▲), 100 mM KCl (♦) or in Tris buffer alone (■). Measurements for determination of inhibition by TCS (B) were performed in 25 mM KPi buffer containing 100 mM NaCl in the absence of TCS/ethanol (▲) or in the presence of 0.5% ethanol [v/v] (●) or 50 μM TCS (■). All experiments were performed at room temperature. Uptake was started by adding 1 mM carrier-free [^{14}C]glycine betaine (0.1 μCi/ml). Each value is the mean ± S.E.M. of at least two independent measurements.

pronounced effect (50% inhibition). Half maximal activity was observed at 11.6 μM GB, and the v_{max} was 317 nmol/min × mg protein (Fig. 5B). GB transport was strictly Na^+ dependent but not inhibited by the sodium ionophore ETH2120, indicating that a Na^+ gradient is not required (Fig. 6A). GB transport was inhibited by the protonophore 3,3′,4′,5-tetrachlorosalicylanilide (TCS) (Fig. 6B) which is consistent with the notion that OpuD3 is a secondary active, membrane potential-driven Na^+:GB symporter.

GB is known as compatible solute synthezised or taken up by bacteria as osmostress response (Hoffmann *et al*., 2013; Scholz *et al*., 2016). Solute transporters of the BCC-type are activated by an osmolarity gradient across the membrane that is sensed by a long C-terminal domain that is assumed to interact with the cytoplasmic membrane (Schiller *et al*., 2004; Schiller *et al*., 2006; Ott *et al*., 2008). This domain is missing in OpuD3. Nevertheless we analysed the effect of an osmolarity gradient on OpuD3-mediated GB transport. Therefore, cells were preincubated in buffer with 100 mM KCl and then the KCl concentration was increased up to 500 mM. The external KCl concentration had no effect on GB transport (data not shown). Taken together, this demonstrates that OpuD3 is not a solute transporter but functions to accumulate the carbon and energy source GB.

OpuD2 is neither a GB importer nor a DMG exporter

The gene encoding OpuD2 was cloned and expressed as described before and the protein was present in the membrane of *E. coli* MKH13-OpuD2, as determined by Western blotting using antibodies against the His-Tag. In contrast to *E. coli* MKH13-OpuD3, *E. coli* MKH13-OpuD2 did not take up radioactively labelled [^{14}C]GB. Transport could also not be activated by an osmolarity gradient. We then speculated that OpuD2 may be a DMG exporter. To test this, inverted membrane vesicles (IMVs) were prepared from *E. coli* MKH13-OpuD2. These IMVs catalysed NADH oxidation and NADH oxidation was coupled to chemiosmotic ATP synthesis, indicating that the IMVs were energetically intact. These IMVs did neither take up DMG when energized with NADH nor under de-energized conditions. DMG uptake could not be driven by artificial ion gradients or GB gradients. In line with the notion that *A. woodii* does not grow on DMG (or sarcosine and glycine), OpuD2 did not take up radiolabeled [^{14}C]DMG in experiments with resting cells of *E. coli* MKH13 at 37°C. The function of OpuD2 remains to be established.

GB is demethylated to DMG

GB carries three methyl groups that each could be used as carbon and energy source. Methyl groups are converted according to:

$$4\,CH_3-X + 2\,CO_2 + 2\,H_2O \rightarrow 3\,CH_3COOH + 4\,H-X$$

Thus, 75% of the methyl groups are converted to the methyl group of acetate. When 20 mM GB (equivalent to 60 mM CH_3-X) was added to resting cells, 13.4 mM acetate were formed, indicating that only one methyl group of GB was used by *A. woodii.* The same ratio was observed at higher GB concentrations, where 27.6, 30.4 and 43.7 mM acetate were formed from 40, 50 and 60 mM GB, respectively. These data indicate that DMG is the product of GB metabolism. Cell suspension did not form acetate from DMG, sarcosine or trimethylamine. The product of GB demethylation was finally determined by thin layer chromatography (TLC). Resting cells of *A. woodii* were supplemented with 50 mM GB and samples were taken over the course of 12 h. Cells were removed by centrifugation and the resulting supernatants were analysed by TLC (Fig. 7). After 1 h the

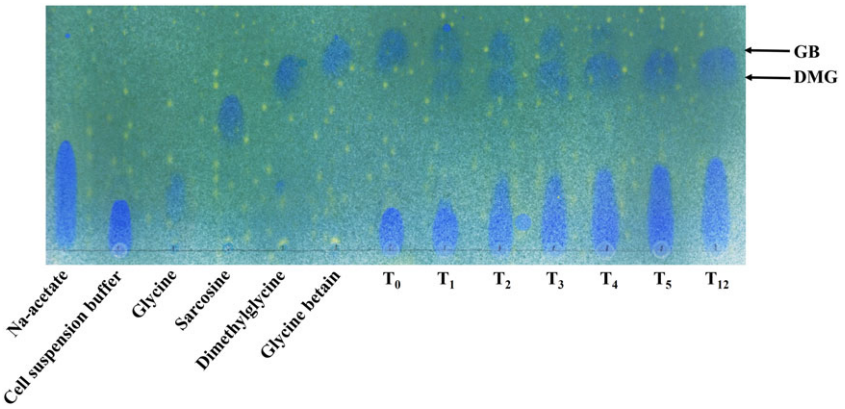

Fig. 7. GB is demethylated to dimethylglycine in resting cells of *A. woodii*. Resting cells of *A. woodii* were incubated at 30°C in a shaking water bath. Samples were taken directly after addition of 50 mM GB (T_0) as well as after 1, 2, 3, 4, 5 and 12 h (T_1–T_{12}). Samples were separated by TLC using 90% phenol in water as mobile phase. GB, dimethylglycine, sarcosine, glycine and Na-acetate were used as standards (1.5 μmol each). TLC-plates were stained with 0.1% bromocresol green. Arrows indicate the migration behaviour of GB and dimethylglycine (DMG).

concentration of GB began to decrease. Simultaneously another compound appeared whose migration behaviour correlated with the one of DMG. The concentration of the compound increased over time whereas the concentration of GB decreased. After 12 h no more GB was detectable in the supernatant. Furthermore sarcosine or glycine could not be detected. These data prove that only one methyl group of GB is used by *A. woodii*, yielding DMG as product of the metabolism. In addition the experiment shows that DMG is excreted by the cells.

GB metabolism requires Na^+ and CO_2/bicarbonate

If surmised that the methyl group of GB is transferred to the WLP at the level of methyl-THF, oxidation of methyl-THF to CO_2 would yield six reducing equivalents which could then reduce three mol of CO_2 to CO. Thus 75% of the methyl groups should end up in the methyl group of acetate and this requires fixation of CO_2. Indeed, acetate formation from methyl groups was strictly dependent on CO_2/bicarbonate (Fig. 8A). This experiment implies that the WLP is involved in methyl group oxidation. Since the WLP is strictly sodium ion-dependent in *A. woodii*, this hypothesis could be tested and indeed, acetogenesis from GB strictly required Na^+ (Fig. 8B).

Discussion

GB metabolism in *A. woodii* involves a methyltransferase system specifically induced by GB. The predicted proteins of the methyltransferase system are similar to MTI, CoP and MTII of other methyltransferase systems of bacteria and methanogenic archaea. Highest similarity is to the biochemically proven GB-demethylating system of *D. hafniense* (Ticak *et al*., 2014; Fig. 9). Thus, we propose that Awo_c07520, Awo_c07540 and Awo_c07550 encode the GB-specific MTII, MTI and CoP, respectively. This hypothesis is corroborated by the notion that Awo_c07560, which is part of the operon, encodes for the GB transporter, OpuD3. Using the amino acid sequence of either MttB10 or OpuD3 in BlastP analysis identified further potential gene clusters in acetogens that might be involved in GB metabolism. In *S. ovata* this approach led to the identification of two distinct gene clusters. Whereas SOV_3c09880-SOV_3c09860 encodes a methyltransferase system with high similarity to the GB-dependent methyltransferase system of *A. woodii*, SOV_3c09320-SOV_3c09090 codes for potential GB-transporters and a betaine specific reductase system. Visser *et al*. identified these proteins of strain An4 in a proteomic approach and proposed a metabolic model in which the methyl group of GB is not directly transferred to THF but GB is reduced to TMA first (Visser *et al*., 2016). Although an additional demethylation of GB was not ruled out, the fate of the resulting methyl group was not determined. Due to the high identity values between the GB-dependent methyltransferase system of *A. woodii* and SOV_3c09880--SOV_3c09860 (Fig. 9), MttA, MttB5 and MttC6 of *S. ovata* might be responsible for direct demethylation of GB and the subsequent methyl transfer to THF. Since TMA and DMG are both produced by *S. ovata* during growth on GB (Möller *et al*., 1984) it is reasonable that the two pathways exist simultaneously. The closely related acetogen *A. dehalogenans* shows the same genetic organization of the GB-specific methyltransferase system as in *A. woodii* (Fig. 9) and identities up to 94%, therefore, it is likely that A3KSDRAFT_02713-A3KSDRAFT_02709 encodes a GB-specific methyltransferase system. Although it is known that *E. limosum* can utilize GB (Müller *et al*., 1981) thereby producing DMG, the BlastP search identified *mttB10* and *opuD3* homologues with only around 30%–40% identity (Eli_3687-Eli_3695 and Eli_2710-Eli_2716, not shown), thus we cannot propose a role in GB metabolism.

OpuD3 produced in *E. coli* MKH 13 restored GB uptake in this strain devoid of endogenous GB transporter. Transport was rather specific for GB, demonstrated by the fact that even a 50-fold molar excess of DMG (50%), sarcosine (31%) and glycine (36%) inhibited GB transport only marginally. Transport was Na^+ dependent and inhibited by a protonophore, supporting the

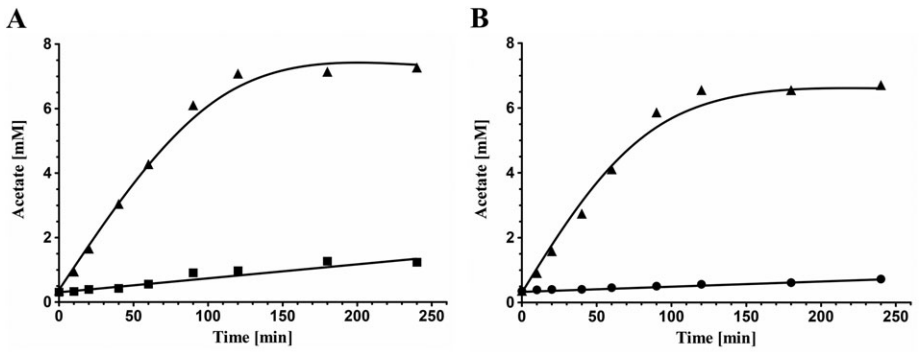

Fig. 8. Acetate formation from GB was strictly dependent on CO_2/bicarbonate and Na^+ in resting cells of *A. woodii*. *A. woodii* was grown on GB (50 mM), harvested, and cells were washed and resuspended to a protein concentration of 1 mg/ml in 20 ml of imidazole buffer in 120-ml serum bottles. The cell suspension experiments were carried out at 30°C in a shaking water bath. The substrate was 10 mM GB. Buffer containing $KHCO_3$ and NaCl was used as positive control (▲). The effect of CO_2/bicarbonate (A) and Na^+ (B) on acetate production was analysed using buffer without $KHCO_3$ (■) or without NaCl (●). The gas phase was N_2-CO_2 (80%/20% [v/v]) except for the experiment where CO_2/bicarbonate dependence was investigated. Here it was 100% N_2. The acetate concentration in samples was determined by gas chromatography.

prediction from the DNA sequence that OpuD3 is a secondary active, Na^+-coupled BCC transporter that is osmolarity independent. The second gene in the operon, Awo_c07530, encodes another potential secondary active BCC transporter with similarities to GB transporters. However, OpuD2 apparently did not transport GB. It also did not export DMG. It also did not catalyse DMG import, an unlikely function anyway, since cells did not grow on DMG. They also did not grow on sarcosine or glycine and thus, a function of OpuD2 as sarcosine and glycine transporter may be excluded. The function of OpuD2 remains to be established. Possible candidates are other quartenary amines such as butyrobetaine, turicine or betonicine but they are not available commercially.

For example, GB is apparently demethylated only to DMG which seems to be a waste of energy. Even after long incubation of several weeks (10), cells did not grow on DMG. This has been observed before for *Eubacterium limosum* (Müller *et al*., 1981). Thus, we have to conclude that only one methyl group of the substrate is used and two thirds of the methyl groups are exported again with DMG. The exporter for DMG remains to be identified. One option may be that OpuD2 is a GB/DMG antiporter. Product/substrate antiporter to mediate substrate import driven by

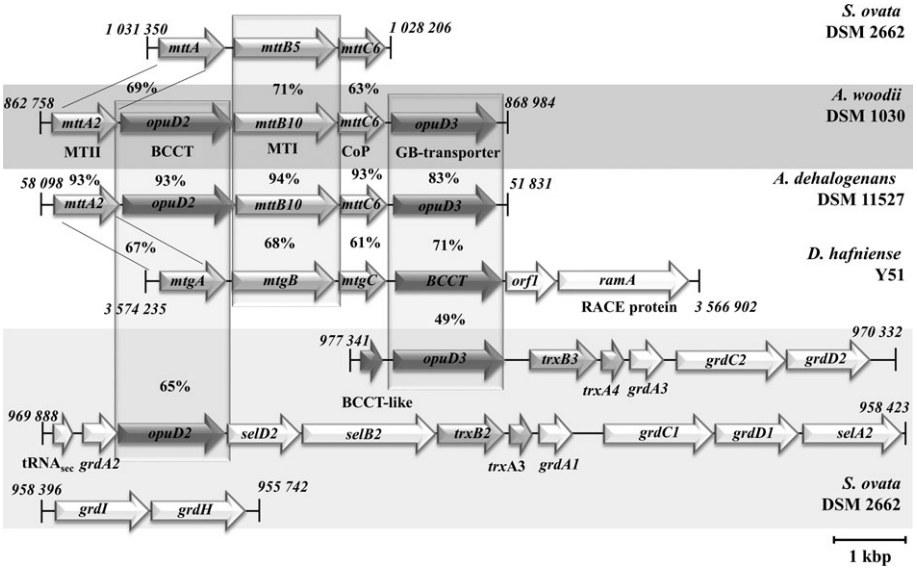

Fig. 9. Genetic organization of known and potential GB clusters. To find potential GB clusters the amino acid sequence of either MttB10 or OpuD3 was used in BlastP searches. Identity on protein level between the selected organisms and *A. woodii* is given in percent. σ^{54}; σ^{54}-dependent transcriptional response regulator, **BCCT**, betaine/choline/carnitine transporter; **MT**, methyltransferase; **CoP**, corrinoid protein; **RACE protein**, reductive activation of corrinoid-dependent enzyme; ***trx***, thioredoxin system components; ***grd***, glycine reductase complex components; ***sel***, selenocystein synthesis components. **Accession no.** are as follows: *S. ovata*; SOV_3c09880-SOV_3c09860 and SOV_3c09320-SOV_3c09090, *A. woodii*; Awo_c07520-Awo_c07560, *A. dehalogenans*; A3KSDRAFT_02713-A3KSDRAFT_02709, *D. hafniense*; DSY3157-DSY3152. The genes shown for *S. ovata* in the lower panel are in one cluster.

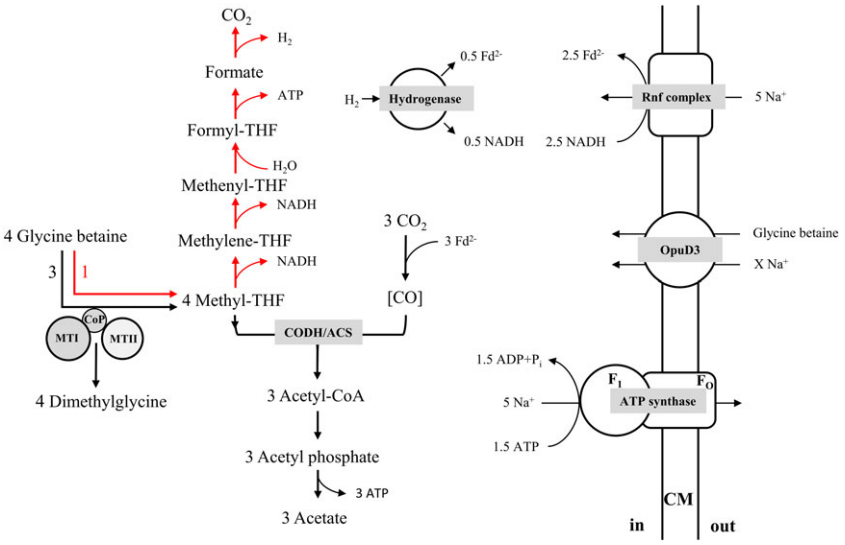

Fig. 10. Carbon and electron flow in GB metabolism of *A. woodii.* The BCC transporter OpuD3 is involved in glycine betaine:Na^+ symport, using an indeterminate number (*X*) of sodium ions. To generate electrons for CO_2 reduction one of four methyl groups has to be oxidized in the methyl branch of WLP. The bifurcating hydrogenase as well as the Rnf complex are used for redox balancing of NADH and Fd. Therefore, a reverse Na^+ flow, energized by ATP hydrolysis, is required. The Na^+/ATP stoichiometry of the ATP synthase is 3.3 (Matthies *et al*., 2014). [Color figure can be viewed at wileyonlinelibrary.com]

product efflux is known in the literature (Poolman *et al*., 1991; Jung *et al*., 2002; Schulze *et al*., 2010). The DMG produced can be taken up as osmoprotectant by, for example, *Bacillus subtilis* (Bashir *et al*., 2014) or catabolized further under aerobic conditions (Wargo *et al*., 2008; Hampel *et al*., 2014).

The methyl group enters the WLP, most likely, at the level of methyl-tetrahydrofolate (Stupperich and Konle, 1993; Kreft and Schink, 1994). The methyl group is then oxidized to CO_2 by a reversal of the WLP, generating six reducing equivalents. Reduction of one mol CO_2 to CO requires two reducing equivalent, and thus, to balance out the electrons, three mol of CO_2 have to be reduced to CO. The overall stoichiometry is

$$4\,GB + 2\,CO_2 \rightarrow 4\,DMG + 3\,Acetate.$$

Figure 10 summarizes the metabolism of GB in *A. woodii.* The methylene-tetrahydrofolate reductase is not electron bifurcating (Bertsch *et al*., 2015; Kremp *et al.*, 2018) and the electron bifurcating hydrogenase as well as the Rnf complex are required to reduce ferredoxin with NADH and H_2 as reductant according to

$$H_2 + 0.5\,NAD^+ + 0.5\,Fd \rightarrow 1.5\,H^+ + 0.5\,NADH + 0.5\,Fd^{2-}$$

$$2.5\,NADH + 5\,Na^+_{out} + 2.5\,Fd \rightarrow 2.5\,NAD^+ + 2.5\,H^+ + 5\,Na^+_{in} + 2.5\,Fd^{2-}.$$

The Rnf complex catalyses reverse electron transport, driven by the electrochemical Na^+ potential established by the ATPase. One mol of ATP is generated *via* substrate level phosphorylation (SLP) in the formyl-tetrahydrofolate synthase reaction during oxidation of the methyl group, while the acetate kinase reaction yields 3 additional mol in the course of methyl group reduction to acetate. Of the resulting total 4 mol of ATP, 1.5 mol have to be invested to export Na^+ to build the membrane potential needed by the Rnf complex to reduce ferredoxin. This would leave 2.5 mol of ATP for the cells to grow. However, since Na^+:GB symport by OpuD3 is Na^+ dependent, some of the remaining ATP is likely to be used for the export of additional sodium ions. Alltogether, 0.83 mol ATP are generated per mol of acetate produced. If Na^+:GB symport is at 1:1 stoichiometry, the ATP yield is lower, 0.73 mol ATP/acetate. If OpuD2 is a substrate:product antiporter, then this would make GB uptake energetically neutral.

Experimental procedures

Organisms and cultivation

A. woodii DSMZ 1030 was routinely cultivated under anaerobic conditions at 30°C in the medium described by Heise and colleagues (1989). For growth experiments concerning the adaption of *A. woodii* to GB, cells were cultivated in minimal medium. This medium was prepared using anaerobic techniques described before (Hungate, 1969; Bryant, 1972) and contained (in g/l): KH_2PO_4, 0.2; NH_4Cl, 1.35; $CaCl_2 \times 2\ H_2O$, 0.11; KCl, 0.5; $MgSO_4 \times 7\ H_2O$, 1.45; NaCl, 1.16; $KHCO_3$, 6.0; cysteine hydrochloride, 0.5 g/l; Calcium D(+)-pantothenate, 1 mg/l; plus selenite-tungstate solution (Tschech and Pfennig, 1984), 1.5 ml/l; vitamin solution DSMZ 141, 2 ml/l and 0.1% [w/v] resazurin solution, 1 ml/l. The medium was gassed with N_2-CO_2 (80%/20% [v/v]) for 20 min before sterilization. Anaerobical and sterile trace element solution SL9 (Tschech and Pfennig, 1984) (5 ml/l) was added afterwards. Different concentrations of either GB, dimethylglycine, sarcosine, trimethylamine, methanol or fructose served as a substrate for *A. woodii.* All cultivations were performed under an N_2-CO_2 (80%/20% [v/v])

atmosphere. *E. coli* MKH13 (Δ*betT*, *putPA*, *prop*, *proU*) (Haardt *et al*., 1995) was grown aerobically in LB medium containing 100 μg/ml ampicillin on a rotary shaker at 37°C. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Analysis of gene expression and operon analysis

Cells of *A. woodii* grown on GB (50 mM), methanol (20 mM) or fructose (20 mM) were harvested in the exponential growth phase at OD_{600} = 0.8–1. Preparation of RNA and sample analysis was essentially as described before (Bertsch *et al*., 2016).

Preparation of cell suspensions

All buffers and the medium were prepared using the anaerobic techniques described previously (Hungate, 1969; Bryant, 1972). All preparation steps of cell suspensions were performed at room temperature under strictly anoxic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) filled with 98% N_2 and 2% H_2.

For cell suspension experiments in which demethylation of GB was analysed, cells of *A. woodii* grown on GB (50 mM) in a volume of 500 ml were harvested in the exponential phase at OD_{600} = 0.6–0.7 (11300 x g, 12 min, 4°C), washed twice in phosphate buffer without CO_2/bicarbonate (30.8 mM K_2HPO_4, 19.2 mM KH_2PO_4, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, dithioerythritol (DTE), 4.4 μM resazurin, pH 7.0) and subsequently resuspended in 0.5 ml of the same buffer. The gas phase of the concentrated cell suspension was changed to 100% N_2.

For cell suspension experiments in which acetate production under different conditions was investigated, cells of *A. woodii* grown on GB (50 mM) in a volume of 500 ml were harvested at OD_{600} = 1 (11 300 × g, 10 min, 4°C), washed three times in imidazole buffer (50 mM imidazole, 60 mM $KHCO_3$, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 4 mM DTE, 5 μM resazurin) and resuspended in 15 ml of the same buffer. For the dependence of acetate production on CO_2/bicarbonate and sodium ions cell suspensions were stored in NaCl- or $KHCO_3$- free imidazole buffer. Total protein concentrations of concentrated cells were determined according to Schmidt *et al*. (1963). Cells were stored at 4°C.

Cell suspension experiments

All experiments were performed at 30°C in a shaking water bath. For cell suspension experiments in which demethylation of GB was investigated, concentrated cells were given to 20 ml phosphate buffer containing CO_2/bicarbonate (30.8 mM K_2HPO_4, 19.2 mM KH_2PO_4, 60 mM $KHCO_3$, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, dithioerythritol (DTE), 4.4 μM resazurin, pH 7.0) in 120-ml serum bottles. The final protein concentration was 1 mg/ml and the gas phase was N_2-CO_2 (80%/20% [v/v]). Prior to the addition of GB (50 mM), the cells were incubated at 30°C for 10 min. Samples (200 μl) were taken directly after the addition of substrate and as specified in the experiment. From this point on samples were treated aerobically. Cells were removed by centrifugation (16 400 × g, 5 min) immediately after sampling and supernatants were stored at 4°C.

For cell suspension experiments in which acetate production under different conditions was investigated, concentrated cells were given to 20 ml imidazole buffer (50 mM imidazole, 60 mM $KHCO_3$, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 4 mM DTE, 5 μM resazurin) in 120-ml serum bottles, the final protein concentration was adjusted to 1 mg/ml. The dependence of acetate production on CO_2/bicarbonate and sodium ions was determined in $KHCO_3$-free (50 mM imidazole, 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 4 mM DTE, 5 μM resazurin) or NaCl-free (50 mM imidazole, 60 mM $KHCO_3$, 20 mM KCl, 20 mM $MgSO_4$, 4 mM DTE, 5 μM resazurin) imidazole buffer. The gas phase was N_2-CO_2 (80%/20% [v/v]) except for the experiment where CO_2/bicarbonate dependence in $KHCO_3$-free imidazole buffer was investigated. Here the gas phase was 100% N_2. Prior to the addition of the substrate, cells were incubated at 30°C for 10 min. Different concentrations of GB, dimethylglycine, sarcosine and trimethylamine served as substrates. Samples (500 μl) were taken as indicated under aerobic and sterile conditions. Cells were removed by centrifugation.

Detection of glycine betaine and demethylation products by thin-layer chromatography

For detection of GB and demethylation products in supernatants of *A. woodii* cell suspensions, 30 μl of supernatants from the cell suspension experiment as well as cell suspension buffer were spotted onto a 10 x 20 cm silica gel 60 thin-layer chromatography (TLC) plate (Macherey-Nagel, Germany). Na-acetate, GB, dimethylglycine, sarcosine and glycine (1.5 μmol each) were used as the standards. Samples were separated using 90% phenol in water (AppliChem GmbH, Germany) as mobile phase. After being dried at room temperature for 2–3 days the plate was stained with 0.1% bromocresol green (in pure ethanol).

Determination of acetate concentrations

Acetate was detected as described previously (Trifunović *et al*., 2016).

Cloning of opuD2 and opuD3

Chromosomal DNA of *A. woodii* was isolated with DNeasy Blood & Tissue Kit (QIAGEN, Netherlands).

The genes *opuD2* and *opuD3* were amplified from 100 ng genomic DNA of *A. woodii* by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Germany) according to the manufacturer's protocol. Restriction sites for SacI and KpnI were also introduced by this PCR (oligonucleotides: sacI_*opuD2*_for: TTAGAGCTCATGCAAGAAAAAACGACAAAGAATCGAACAGTGTTGTATG and kpnI_*opuD2*_rev: TTTGGTACCTTATTCTTGAACATCCACAACTGGCGTTTGTCCGTTGTC for cloning of *opuD2*; sacI_*opuD3*_for: TTTGAGCTCATGGTATTTTACGTTTCAATTATACTTATTC and kpnI_*opuD3*_rev: TTTGGTACCCTAACGTTTACTTTCATTTTTTAAAGCCAAATATAGTG for cloning of *opuD3*, underlined sequences indicate restriction sites). PCR fragments were cloned into pBAD/His (Invitrogen, Germany). The constructs were verified by DNA sequencing.

Protein production

The vectors pBAD/His, pBAD/His_*opuD2* or pBAD/His_*opuD3* were transformed into *E. coli* MKH13 by the method described by Chung *et al*. (1989). Cells containing the corresponding vectors were cultivated at 37°C in LB medium containing 100 μg/ml ampicillin. Gene expression was induced at an OD_{600} of 0.7–0.8 by addition of L-arabinose to a final concentration of 0.02%. After 2.5 h at 37°C the cells were harvested (4200 × g, 10 min).

[^{14}C]glycine betaine and [^{14}C]dimethylglycine uptake in E. coli *MKH13*

Protein production in cells of *E. coli* MKH13 transformed with either pBAD/His, pBAD/His_*opuD2* or pBAD/His_*opuD3* was carried out as described above. For detecting GB or dimethylglycine uptake, Km value determination and competition experiments cells were washed twice in 25 mM KPi buffer (20.35 mM K_2HPO_4, 4.65 mM KH_2PO_4, pH 7.5) containing 100 mM NaCl and resuspended in the same buffer containing 30 mM glucose to an OD_{600} of 3. Subsequently cells were diluted 1:1 with KPi buffer containing 100 mM NaCl. For detecting GB uptake at different osmolarities cells were washed twice in 25 mM KPi buffer (20.35 mM K_2HPO_4, 4.65 mM KH_2PO_4, pH 7.5) containing 100 mM NaCl and resuspended in the same buffer containing 30 mM glucose to an OD_{600} of 3. Subsequently cells were diluted 1:1 with 25 mM KPi buffer (20.35 mM K_2HPO_4, 4.65 mM KH_2PO_4, pH 7.5) containing 100 mM NaCl and 0–1 M KCl. Final concentrations of KCl in diluted cell suspension were 0, 100, 200, 400 or 500 mM. To determine Na^+-dependence of GB uptake cells were washed twice in 25 mM Tris–HCl buffer (Na^+-free, pH 7.5) containing either 100 mM NaCl or 100 mM KCl and resuspended in the same buffer containing 30 mM glucose to an OD_{600} of 3. Subsequently cells were diluted 1:1 with the corresponding Tris buffer. Total protein concentration of cells was determined according to Schmidt and colleagues (1963).

Diluted cell suspensions were incubated for 3 min at 37°C or room temperature before the addition of 1 mM carrier-free [^{14}C]glycine betaine or [^{14}C]dimethylglycine (each 0.1 μCi/ml). Cell samples were taken at various intervals, passed through 0.45 μm nitrocellulose membrane filters and washed twice with the 12.5-fold amount of the corresponding buffer. An exception here is the measurements at different osmolarities, where 0.6 M KPi buffer (488 mM K_2HPO_4, 112 mM KH_2PO_4, pH 7.5) was used for washing of membrane filters. Radioactivity retained on the filters was quantified by liquid scintillation counting. Therefore, the filters were dried, and solved in scintillation reagent (Rotiszint eco plus, Carl Roth GmbH & KG, Germany). Radioactivity was then determined by a liquid scintillation counter (Wallace, 1409 DAS, PerkinElmer, USA).

Preparation of inverted membrane vesicles

Protein production in cells of *E. coli* MKH13 transformed with pBAD/His_*opuD2* was carried out as described above in a culture volume of 2 l. Afterwards the cells were harvested and washed once in 50 mM Tris (pH 8) buffer containing 300 mM NaCl. Cell pellets were shock frozen in liquid nitrogen and stored at −70°C until use. The cells were resuspended in 25 ml TMNGD buffer (50 mM Tris, 25 mM $MgCl_2$, 25 mM NaCl, 10% [v/v] glycerol, 1 mM DTE, pH 7.5). Depending on the experiment, the buffer contained 1 mM or 1 M GB. After the addition of phenylmethylsulfonyl fluoride (PMSF) (0.5 mM) and DNaseI the cells were once passed through a French press (SLM Aminco, SLM Instruments, USA) at 6300 psi. Cell debris and whole cells were removed by a centrifugation step (20 000 × g, 15 min, 4°C) and the supernatant was centrifuged for 1 h at 180 000 × g and 4°C. Subsequently membrane vesicles were resuspended in 500 μl TMNGD buffer containing 0.1 mM PMSF. The protein concentration of membrane vesicles was determined according to the method of Bradford (Bradford, 1976).

Determination of NADH oxidation and ATP synthesis by inverted membrane vesicles

NADH oxidation was measured photometrically at 340 nm. All measurements were carried out at 37°C. 100 μg of IMV were given to 1 ml ATP synthesis buffer (25 mM Tris, 25 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM NaCl, 10% [v/v] glycerol, pH 7.5) with or without 10 μM TCS followed by a preincubation of 10 min at 37°C. Subsequently basal absorption was followed for 50 s before the reaction was started by addition of 0.5 mM NADH. Decrease of absorption was followed for additional 130 s. ATP synthesis by IMV of

E. coli MKH13-OpuD2 was determined as described before (Gloger *et al*., 2015).

[^{14}C]dimethylglycine uptake in inverted membrane vesicles of E. coli *MKH13*

IMV of *E. coli* MKH13 were prepared as described above and adjusted to a final protein concentration of 1 mg/ml in 1 ml TMGD buffer (50 mM Tris, 25 mM $MgCl_2$, 10% [v/v] glycerol, 1 mM DTE, pH 7.5). The IMV were preincubated for 2 min at 37°C. If vesicles should be energized, NADH (1 mM) was added after preincubation, followed by 2 additional minutes at 37°C. Uptake was started by addition of 1 mM carrier-free [^{14}C]dimethylglycine (0.1 μCi/ml). Samples were taken at various intervals, passed through 0.22 μm nitrocellulose membrane filters and washed twice with the 12.5-fold amount of TMGD buffer. Radioactivity retained on the filters was quantified by liquid scintillation counting. Therefore, the filters were dried, and solved in scintillation reagent (Rotiszint eco plus, Carl Roth GmbH & KG, Germany). Radioactivity was then determined by a liquid scintillation counter (Wallace, 1409 DAS, PerkinElmer).

Immunological methods

For immunological detection of His-OpuD2 and His-OpuD3, the proteins were produced in *E. coli* MKH13. Cells were disrupted by one passage through a French press (Aminco, SLM Instruments, USA) at 16 000 psi. Cell debris was removed by centrifugation. 25 μg of protein from the supernatant were separated on a 12.5% polyacrylamide gel according to the method of Schägger and von Jagow (1987), proteins were transferred to a nitrocellulose membrane (Amersham Protran 0.2 μm NC, GE Healthcare Life Sciences, United Kingdom) followed by immunoblotting using Penta-His Antibody, BSA-free (QIAGEN, Netherlands) as a primary antibody with a 1:1000 dilution and goat anti-mouse IGG (H + L)-horseradish peroxidase conjugate (Bio-Rad Laboratories, USA) as the secondary antibody with a 1:10 000 dilution. Signal detection was performed as described before (Schmidt *et al*., 2007).

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Supporting Information

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

Fig. S1. Growth of *A. woodii* on glycine betaine. The cultures contained either 50 mM glycine betaine (■), dimethylglycine (▲), sarcosine (▼), trimethylamine (♦) or no substrate (●). The optical density at 600 nm was measured over time directly in Hungate tubes without dilution. All cultures were inoculated with glycine betaine-adapted precultures. Each value is the mean ± S.E.M. of three independent measurements.

Table S1. Primers used in this study. Restriction sites of endonucleases are underlined.