

# The Dnmt2 RNA methyltransferase homolog of *Geobacter sulfurreducens* specifically methylates tRNA-Glu

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## ABSTRACT

Dnmt2 enzymes are conserved in eukaryotes, where they methylate C38 of tRNA-Asp with high activity. Here, the activity of one of the very few prokaryotic Dnmt2 homologs from *Geobacter* species (GsDnmt2) was investigated. GsDnmt2 was observed to methylate tRNA-Asp from flies and mice. Unexpectedly, it had only a weak activity toward its matching *Geobacter* tRNA-Asp, but methylated *Geobacter* tRNA-Glu with good activity. In agreement with this result, we show that tRNA-Glu is methylated in *Geobacter* while the methylation is absent in tRNA-Asp. The activities of Dnmt2 enzymes from *Homo sapiens*, *Drosophila melanogaster*, *Schizosaccharomyces pombe* and *Dictyostelium discoideum* for methylation of the *Geobacter* tRNA-Asp and tRNA-Glu were determined showing that all these Dnmt2s preferentially methylate tRNA-Asp. Hence, the GsDnmt2 enzyme has a swapped transfer ribonucleic acid (tRNA) specificity. By comparing the different tRNAs, a characteristic sequence pattern was identified in the variable loop of all preferred tRNA substrates. An exchange of two nucleotides in the variable loop of murine tRNA-Asp converted it to the corresponding variable loop of tRNA-Glu and led to a strong reduction of GsDnmt2 activity. Interestingly, the same loss of activity was observed with human DNMT2, indicating that the variable loop functions

as a specificity determinant in tRNA recognition of Dnmt2 enzymes.

## INTRODUCTION

Several chemical modifications of the bases in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have been discovered in the last decades of research, which include methylation, pseudouridylation, hydroxymethylation, formylation and carboxylation (1–4). In eukaryotes, three families of enzymes with strong sequence similarity to DNA-(cytosine C5)-methyltransferases have been found, namely Dnmt1, Dnmt2 and Dnmt3 (5–7). Dnmt2 enzymes are highly conserved with homologs present from lower eukaryotes like *Schizosaccharomyces pombe* to higher eukaryotes like *Homo sapiens* (8,9). These enzymes contain all motifs characteristic for DNA methyltransferases, but lack the large N-terminal domain found in the Dnmt1 and Dnmt3 enzymes. Later, in a seminal paper by Goll *et al.*, Dnmt2 enzymes were reported to have a robust methylation activity on tRNA<sup>ASP</sup> at position C38 (6). After the initial discovery of tRNA<sup>ASP</sup> methylation by Dnmt2, many studies have confirmed this activity in different organisms (10–16). However, even though Dnmt2 methylates transfer RNA (tRNA), it utilizes a DNA methyltransferase-like catalytic mechanism in the methyl transfer reactions (10), which also explains the conservation of the corresponding amino acid motifs in the catalytic center.

Dnmt2 knockout studies have indicated a connection to stress response and methylation of small RNAs (13,17,18), non-random sister chromatid segregation in stem cells

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(19), mobile element and RNA virus control in *Drosophila melanogaster* (20,21), paramutation in mice (22) and adaptation to different growth conditions in lower eukaryotes (12,15,16). However, it is still unclear whether these effects stem from the loss of methylation of C38 in tRNA<sup>Asp</sup> or whether other Dnmt2 targets are responsible for the phenotypes. Many of the studies have also investigated methylation of alternate tRNA substrates by Dnmt2. In *Dicystostelium discoideum*, Dnmt2 was reported to methylate tRNA<sup>Glu</sup>(CUC/UUC) and tRNA<sup>Gly</sup>(GCC) very weakly *in vitro* (15). The *S. pombe* Dnmt2 homolog, Pmt1, was shown to have a weaker methylation activity on tRNA<sup>Glu</sup> as well (16). *D. melanogaster* Dnmt2 was found to methylate tRNA<sup>Val</sup>(AAC) and tRNA<sup>Gly</sup>(GCC) *in vivo* in addition to tRNA<sup>Asp</sup> (13). However, in all enzymatic studies, tRNA<sup>Asp</sup> was the most preferred substrate for Dnmt2. The molecular basis for the specific interaction of Dnmt2 with its tRNA substrates is not yet known. Previously, we have mapped the binding of tRNA<sup>Asp</sup> to human DNMT2 by mutating several conserved lysine and arginine residues in human DNMT2 and concluded that the anticodon stem/loop of tRNA<sup>Asp</sup> is a main region of contact for human DNMT2 for the methyl transfer reaction by human DNMT2 (23). Supporting this notion, Muller *et al.* proposed a role of the C32, A37 and C40 nucleotides in tRNA recognition (15).

A recent phylogenetic analysis revealed that the *Dnmt2* gene family was most likely derived from a prokaryotic DNA-(cytosine C5)-methyltransferase (9). As mentioned above, the enzyme is highly conserved in all eukaryotic phyla. Strikingly, there are only a few putative Dnmt2 homologs in bacteria, one of which is found in *Geobacter* species (6,9,24), another putative representative is present in *Holophaga foetida* (Supplementary Figure S1). These enzymes are clearly defined by the presence of a CFT amino acid motif (CFI in *Holophaga*), which is characteristic for Dnmt2 enzymes and involved in the tRNA interaction (9,10). A bioinformatics analysis indicated that the *Geobacter* Dnmt2 enzyme is likely the outcome of a horizontal gene transfer from eukaryotes to *Geobacter* (9), which is known for its ability of lateral gene transfer (25,26). This conclusion is supported by the additional identification of a putative Dnmt2 homolog in *Holophaga*, because these bacteria are not closely related to *Geobacter* (*Holophaga* is a member of the Fibrobacteres/Acidobacteria group while *Geobacter* belongs to the  $\delta$ -Proteobacteria).

As part of a larger initiative aiming to investigate the function of Dnmt2 in many species including humans, mice, flies and unicellular eukaryotes and to define its biological role, we set out to investigate the biochemical properties and substrate recognition of *Geobacter sulfurreducens* Dnmt2 (GsDnmt2). Since it has been shown that eukaryotic Dnmt2 enzymes are specific for tRNA<sup>Asp</sup> C38 methylation, our work was started in this direction. However, we surprisingly found that GsDnmt2 specifically methylates *Geobacter* tRNA<sup>Glu</sup> *in vitro* and *in vivo* and not *Geobacter* tRNA<sup>Asp</sup>. This represents the first example of a Dnmt2 enzyme with a completely switched substrate specificity. Based on this finding, we further identify the variable loop as a sequence determinant in tRNA recognition of *Geobacter* Dnmt2 and the human enzyme.

## MATERIALS AND METHODS

### Cloning and site-directed mutagenesis

The gene for *G. sulfurreducens*, GsDnmt2 (GSU0227), was amplified from genomic DNA of *G. sulfurreducens* obtained from DSMZ (Braunschweig, Germany) using a primer set flanking the gene (forward 5'-GCC GCA TAT GAG GGC GGT CGA GCT CTT CTG-3' and reverse 5'-GAT CGG AAT TCT CAC CCC TCC TCA GCC GGT AAC-3'). The amplified gene was cloned into pET28a(+) using NdeI and EcoRI sites and successful cloning validated by DNA sequencing. The catalytic site variant C74A of GsDnmt2 was created by the megaprimer site-directed mutagenesis method as described previously (27). This residue corresponds to C79 in human DNMT2, which is a key catalytic residue, mutation of which has been shown to inactivate the enzyme (10). The primer used for the site-directed mutagenesis was 5'-GTA AGG CTG CGC AGG AGG CGA C-3'. The presence of the desired mutation was confirmed by sequencing. Constructs for other Dnmt2s are as described: human DNMT2 (28), *S. pombe* Pmt1 (16) and *D. discoideum* DnmA (15). The gene for *D. melanogaster* Dnmt2 was amplified from complementary DNA (cDNA) using the following primer pair: 5'-GCG GTG GTG CTC GAG TTA TTT TAT CGT CAG-3' and 5'-GCG GCA GCC ATA TGG TAT TTC GGG TCT TAG AA-3', and cloned into pET28a+ using XhoI and NdeI sites. The cDNA was prepared using RNA isolated from 0–6-h-old embryos of *D. melanogaster* BerlinWild strain. The cloned sequence was found to contain an insertion present in the Isoform A of the *D. melanogaster* Dnmt2 gene, which was subsequently excised by polymerase chain reaction (PCR) mutagenesis performed with the following primers: 5'-ATG CCC AAT TGG ATG GAC AAA TAG TTG CCG CCT TGG-3' and 5'-ACT AAA TAG TTC TAA GAC CCG AAA TAC CAT-3'.

### Protein expression and purification

The wild-type and mutant C74A GsDnmt2 were expressed as His<sub>6</sub>-tagged proteins in *Escherichia coli* Rosetta 2 (DE3) cells. Protein expression and purification was performed as described previously (28). Briefly, transformed Rosetta 2 (DE3) cells were grown to OD<sub>600</sub> = 0.6. Protein expression was induced with 1-mM IPTG (Isopropyl- $\beta$ -D-1-thiogalactopyranosid) and cells were further incubated at 37°C for 3 h. The cells were sonicated in lysis buffer (30-mM potassium phosphate pH 7.5, 500-mM KCl, 0.1-mM DTT (dithiothreitol), 10-mM imidazole and 10% glycerol). Recombinant proteins were purified on Ni-NTA beads (Genoxon) and eluted with 200-mM imidazole in lysis buffer. The protein was then dialyzed against dialysis buffer I (30-mM potassium phosphate pH 7.5, 300-mM KCl, 10% glycerol, 1-mM ethylenediaminetetraacetic acid (EDTA) and 0.1-mM DTT) and dialysis buffer II (30-mM potassium phosphate pH 7.5, 100-mM KCl, 60% glycerol, 1-mM EDTA and 0.1-mM DTT). A similar procedure was followed for human DNMT2 purification. For *D. melanogaster* Dnmt2, the protein expression was conducted at 28°C. For the DnmA recombinant protein, expression was carried out at 22°C. The amounts of all recombinant

proteins were determined by their specific absorbance and the concentrations and purities were verified by Coomassie-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

### *In vitro* transcription of tRNA substrates

The tRNA sequences were derived from the Genomic tRNA data base (<http://gtrnadb.ucsc.edu/>) (29). The tRNA substrates for methylation reactions were prepared by *in vitro* transcription basically as described (10). The DNA templates for the *in vitro* transcription were purchased from MWG. The template DNA and primer sequences are given in Supplemental Table S2. The templates were amplified by PCR with T7 and tRNA-specific primers and the yield and purity of the PCR was inspected on a native 8% acrylamide-TPE (Tris-Phosphate-EDTA) gels. *In vitro* transcriptions were performed in a total volume of 200  $\mu$ l containing 50  $\mu$ l of 4x transcription buffer (200-mM Tris-HCl pH 8.1, 5-mM spermidine, 25-mM DTT, 0.05% TritonX-100, 50-mM MgCl<sub>2</sub> and 0.2-mg/ml bovine serum albumin), 20-mM NTPs (nucleoside triphosphates) mix, 1 U/ $\mu$ l of RNasin plus (Promega), 1 U/ $\mu$ l of T7 RNA polymerase (Fermentas) and 35  $\mu$ l of PCR-amplified template DNA. The reaction was incubated at 37°C for at least 3 h, followed by addition of 15 U DNase I and incubation for another 30 min at 37°C. Then tRNAs were separated on a 12% acrylamide/7-M urea gel and the tRNA bands were excised from the gel (Figure 1B). The tRNA was eluted with elution buffer (50-mM Tris-HCl pH 7.5, 300-mM sodium acetate and 0.5% SDS) overnight at room temperature followed by ethanol precipitation.

### *In vitro* methylation kinetics

The *in vitro* methylation reactions of tRNAs were carried out as described before (30) with few modifications. Before the methylation reactions, the substrate tRNAs were refolded by heating to 65°C and slowly cooled to ambient temperature in the presence of 2-mM MgCl<sub>2</sub> and 2 U/ $\mu$ l of RNasin plus (Promega). The subsequent methylation reaction was performed with 0.5  $\mu$ M of the tRNA substrates and 1  $\mu$ M of the Dnmt2 enzyme in methylation buffer (20-mM Tris-HCl (pH 8), 20-mM NH<sub>4</sub>OAc, 2-mM MgCl<sub>2</sub>, 2-mM DTT and 0.02-mM EDTA). The reactions were started by addition of 0.76- $\mu$ M [methyl <sup>3</sup>H]-AdoMet (Perkin Elmer) and carried out at 22°C with DnmA, 30°C with *Drosophila* Dnmt2 and 37°C with all other enzymes. The reactions were stopped at different time points by removing 5- $\mu$ l aliquots and pipetting them into 500  $\mu$ l of 5% trichloroacetic acid (TCA). The samples were then spotted onto a DE81 anion exchange filter paper disc (0.20 mm thick and 1.5 cm in diameter) and the unbound radioactive AdoMet was washed away with 5% TCA and absolute ethanol. The discs were then air dried and the bound radioactivity was measured with a Hidex 300 SL liquid scintillation counter (Hidex, Finland) using Rotiszint eco plus (Roth, Germany). The reaction progress curves of the single turnover kinetics were fitted using Equation (1), which is derived from a single exponential model,

$$\text{CMP}_{\text{theo}}(t) = f(1 - e^{-k_1 t}) \quad (1)$$

with  $k_1$  being the single turnover rate constant of RNA methylation [ $\text{min}^{-1}$ ] and  $f$  being the saturation level of complete methylation (CPM). To obtain the initial rate of RNA methylation in CMP/min ( $v_0$ ), Equation (1) was differentiated at  $t = 0$ , which results in Equation (2):

$$v_0 = f \times k_1 \quad (2)$$

In many reactions with less active enzyme/substrates combinations a clear linear initial phase was observed. Then, the initial rate constant of RNA methylation was derived directly by linear regression of the reaction progress curve.

### tRNA binding analysis

The tRNA-binding analysis was performed using 3'-end-pCp-labeled tRNAs. For labeling, 1  $\mu$ g of the *in vitro* transcribed Gs-tRNA<sup>Asp</sup> and Gs-tRNA<sup>Glu</sup> was incubated with 5  $\mu$ l [<sup>32</sup>P] Cytidine 3',5'-bisphosphate (pCp) (Hartmann Analytic) and T4 RNA ligase (NEB) in 50  $\mu$ l ligation buffer (50-mM Tris-HCl pH 7.5, 10-mM MgCl<sub>2</sub>, 1-mM DTT and 1-mM adenosine triphosphate) and 10% DMSO (dimethyl sulfoxide) overnight at 4°C. The labeled tRNAs were purified from unincorporated nucleotides by using Micro-Bio-Spin 6 columns (BioRad). The purified tRNAs were refolded and incubated with increasing Dnmt2 concentrations in methylation buffer. After 30 min the reactions were spotted onto a nitrocellulose membrane and washed three times with the reaction buffer. The membranes were exposed to X-ray films and the intensity of the spots was measured using ImageJ software. Data were fitted using the Excel solver module to an equation describing a bimolecular binding equilibrium.

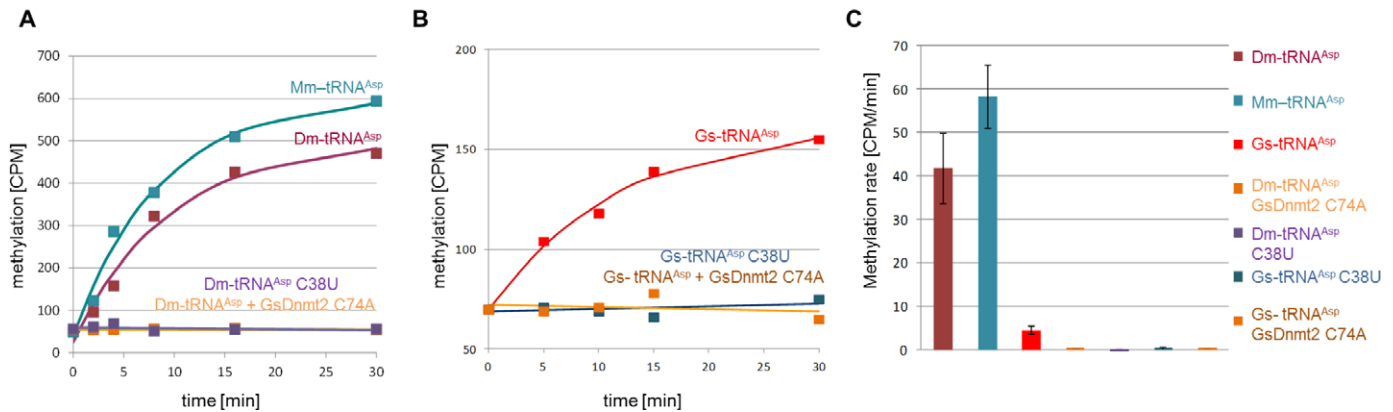
### Total RNA isolation from *G. sulfurreducens*

*G. sulfurreducens* was grown anaerobically (N<sub>2</sub>:CO<sub>2</sub> 80:20) at 30°C in NBAF medium with acetate (15 mM) as the electron donor and fumarate (40 mM) as the electron acceptor as previously described (31). Total RNA isolation from *G. sulfurreducens* was carried out with the MasterPure RNA purification kit (Epicentre) following the manufacturer's instructions. The isolated total RNA was treated with DNase I to remove contaminant genomic DNA. RNA concentrations were determined by ultraviolet spectroscopy.

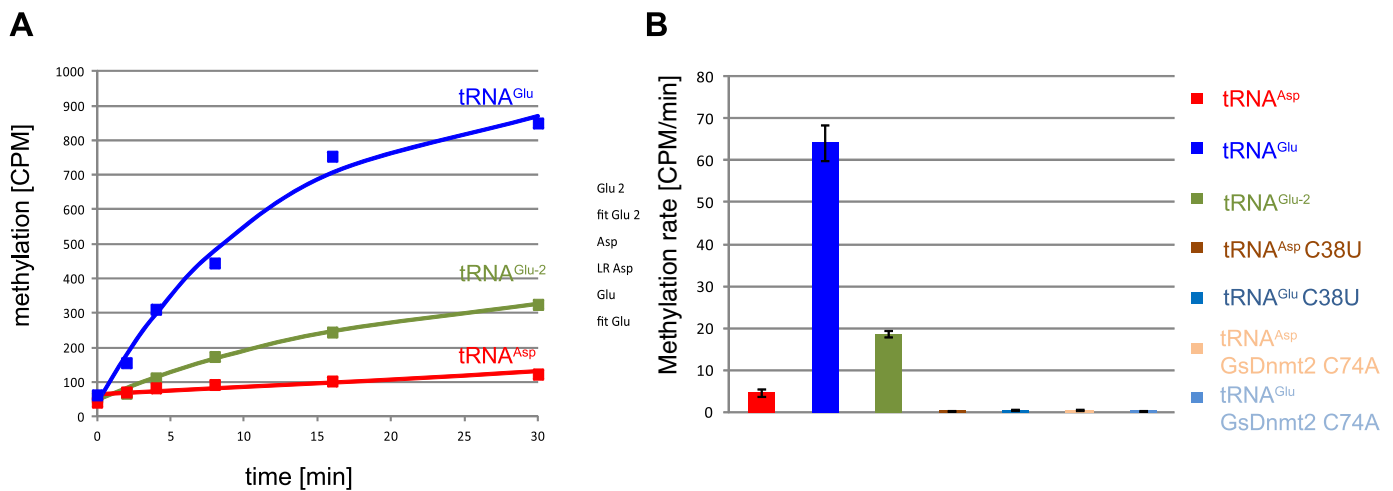
### tRNA bisulfite sequencing

The tRNA bisulfite sequencing was performed as described previously (32). For this study, tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup>, tRNA<sup>Glu-2</sup> and tRNA<sup>Val</sup> were selected because they contain cytosine at position 38 in their sequence. Briefly, 3  $\mu$ g of total RNA was treated with bisulfite following the instructions of the manufacturer (EpiTect Bisulfite Kit, Qiagen). The bisulfite-treated RNA was later used to generate a cDNA by reverse transcription using a stem loop primer (5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGG TAA CAA AATC-3', 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGG TAA TAA TCCC-3' and 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGG TAA





**Figure 1.** *G. sulfurreducens* Dnmt2 methylates C38 of tRNA<sup>Asp</sup>. (A) Methylation of *D. melanogaster* tRNA<sup>Asp</sup> and murine-tRNA<sup>Asp</sup> with the GsDnmt2 enzyme. Control reactions using the catalytically inactive GsDnmt2 mutant C74A and Dm-tRNA<sup>Asp</sup> C38U mutant show no methylation. (B) Methylation of tRNA<sup>Asp</sup> from *G. sulfurreducens* with GsDnmt2 showing weak methylation. Control reactions with the C38U mutant of the tRNA<sup>Asp</sup> or the catalytically inactive C74A variant of GsDnmt2 showed no methylation signal. (C) Summary of the tRNA methylation data reporting average methylation rates and SEM taken from two to three independent experiments.



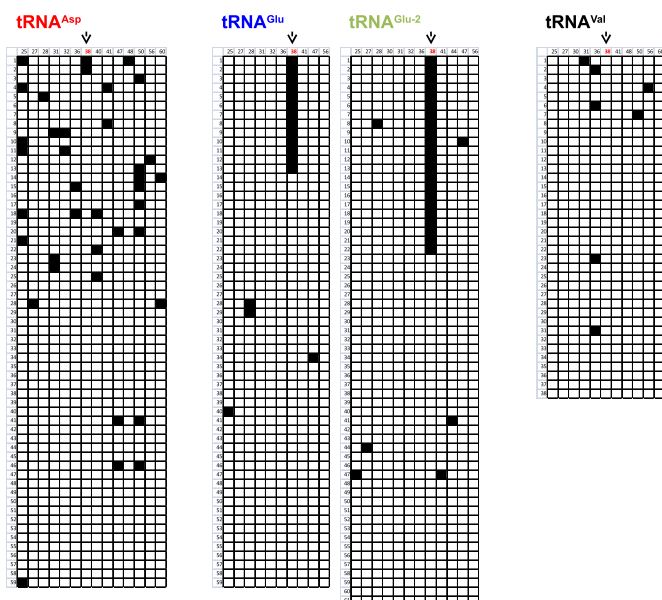
**Figure 2.** GsDnmt2 prefers methylation of *G. sulfurreducens* tRNA<sup>Glu</sup> over tRNA<sup>Asp</sup>. (A) Examples of *in vitro* methylation of *Geobacter* tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> by GsDnmt2. (B) Average methylation rates and SEM based on three repeats of the experiments. Panel (B) also includes results of control reactions with tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> C38U variants and with the catalytically inactive C74A GsDnmt2 protein. The error bars indicate the SEM from at least three different experimental repeats.

TAA ACAC -3') for tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup> and tRNA<sup>Val</sup>, respectively. The reverse transcriptase-PCR product was further amplified by PCR using a universal stem loop primer 5'-CAC GAC ACC AGT TGA-3' and deaminated tRNA-specific primers with the following sequence: 5'-TAG TTA AGT TGG TTAT-3', 5'-GTT TAG TGG TTA GGA-3' and 5'-TTA GTT TAG TGG GAGA-3' for tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup> and tRNA<sup>Val</sup>, respectively. The PCR products were cloned by TOPO-TA cloning (Invitrogen, Germany). Colony PCR was performed to confirm the presence of desired inserts. To determine the RNA methylation, 60 clones were sequenced for each tRNA (33).

## RESULTS

### GsDnmt2 is an active enzyme capable of methylating C38 in tRNA<sup>Asp</sup>

After successfully cloning the *GsDnmt2* gene into the pET28a+ vector, expressing and purifying the protein (Supplementary Figure S3), its activity was tested using *in vitro* transcribed tRNAs (Supplementary Figure S4). It was known from many previous reports that Dnmt2 from all the eukaryotic organisms methylate tRNA<sup>Asp</sup> (6,10,12–16). To test whether the bacterial Dnmt2 also methylates tRNA<sup>Asp</sup>, *in vitro* transcribed tRNA<sup>Asp</sup> of *D. melanogaster* was used as the initial substrate. Since the tRNA methylation rate of human DNMT2 is in the range of few turnovers per hour (10) and the Dnmt2 enzymes from other species studied here showed similar or lower activity, it was not possible to conduct steady-state methylation experiments. Therefore, we followed the initial time course of tRNA methylation re-



**Figure 3.** tRNA<sup>Glu</sup> but not tRNA<sup>Asp</sup> is methylated at C38 in *G. sulfurreducens*. The figure shows results of RNA bisulfite sequencing of tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup>, tRNA<sup>Glu-2</sup> and tRNA<sup>Val</sup> from total RNA isolated from *G. sulfurreducens*. The cytosine residues present in the corresponding tRNA are indicated in the top row. Each subsequent row represents an independent clone that was sequenced. Black boxes indicate methylated cytosines and white boxes indicate unmethylated cytosines. The arrows highlight the C38 positions.

actions under single turnover conditions using excess of enzyme. As shown in Figure 1A and C, wild-type GsDnmt2 methylated tRNA<sup>Asp</sup> while no activity was detected with the active site mutant (C74A) in which the catalytic cysteine 74 residue, which is located in a highly conserved PCQ motif in Dnmts, was altered to alanine. The corresponding exchange in human DNMT2 has been shown to inactivate the enzyme as well (10). The observation that the C74A variant lost the methylation activity indicates that GsDnmt2 is an active tRNA methyltransferase and capable of methylating *in vitro* transcribed *D. melanogaster* tRNA<sup>Asp</sup>. The next aim was to confirm that the site of methylation by GsDnmt2 is the same as by other members of the Dnmt2 family. For this, a C38U mutant of the *D. melanogaster* tRNA<sup>Asp</sup> was prepared and methylation reactions were performed with the wild-type GsDnmt2 enzyme. A complete loss of methylation was observed with this mutant tRNA, indicating that cytosine 38 is the methylation target site for GsDnmt2. Finally, methylation of murine tRNA<sup>Asp</sup> by GsDnmt2 was demonstrated as well. In summary, these data indicate that GsDnmt2 methylates tRNA<sup>Asp</sup> from *D. melanogaster* and *M. musculus* at C38.

#### GsDnmt2 is weakly active on *Geobacter*-encoded tRNA<sup>Asp</sup>

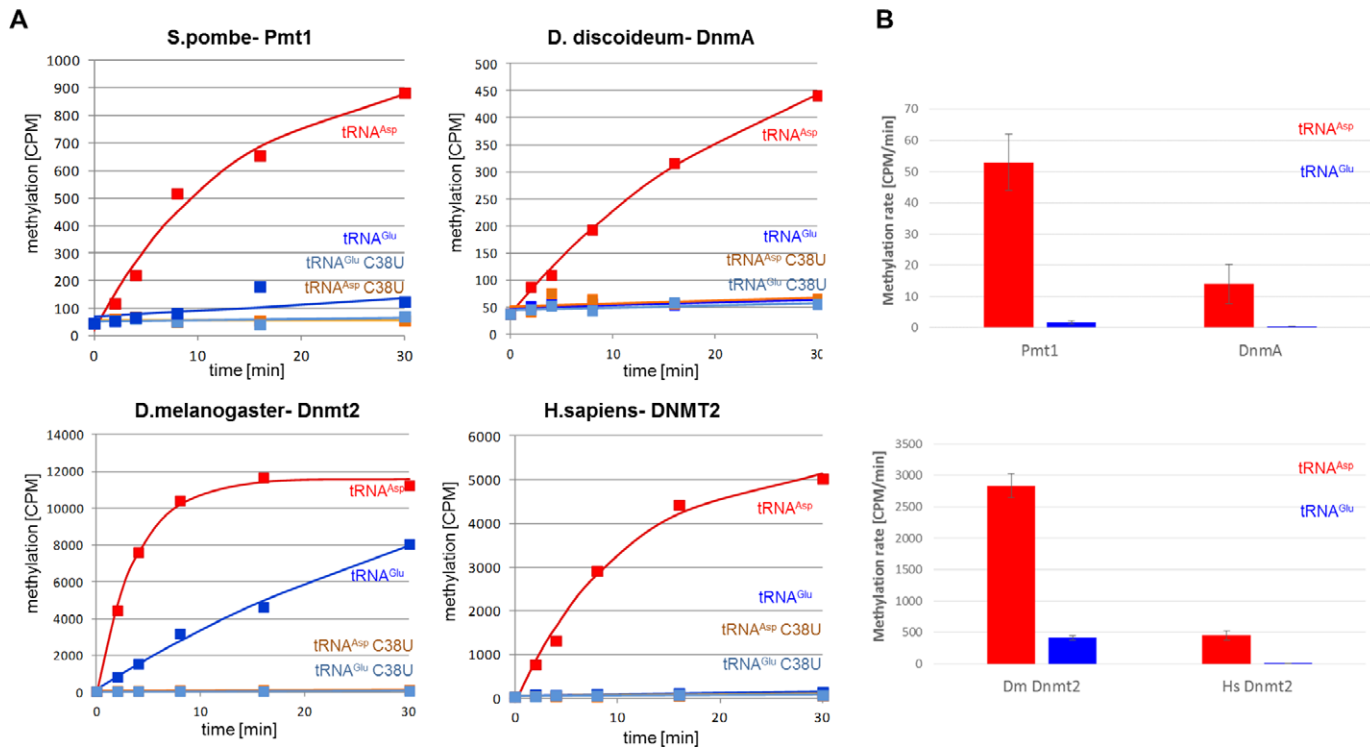
After confirmation of the activity of GsDnmt2 on tRNA<sup>Asp</sup> derived from flies and mice, the catalytic activity of this enzyme on *Geobacter*-encoded tRNA<sup>Asp</sup> was investigated, which shares large similarity with eukaryotic tRNA<sup>Asp</sup> (Supplementary Figure S2). For this, *G. sulfurreducens* tRNA<sup>Asp</sup> (Gs-tRNA<sup>Asp</sup>) was prepared by *in vitro* transcrip-

tion and an *in vitro* methylation was performed using the recombinantly expressed wild-type GsDnmt2. However, as shown in Figure 1B and C, GsDnmt2 was only weakly active on its cognate tRNA<sup>Asp</sup>. No activity was observed with the C74A mutant protein, confirming that the radioactive signal observed was due to tRNA methylation by wild-type GsDnmt2. In addition, the site of methylation was also confirmed to be cytosine 38 in Gs-tRNA<sup>Asp</sup> by C38U mutation. The weak methylation of Gs-tRNA<sup>Asp</sup> was not related to the preparation of the tRNA, because the same tRNA preparation was efficiently methylated by Dnmt2 enzymes from other species (see below, Figure 4). In summary, when comparing methylation of tRNA<sup>Asp</sup> from flies, mice and *Geobacter*, GsDnmt2 unexpectedly showed the weakest methylation activity with its own *Geobacter*-encoded tRNA<sup>Asp</sup>.

#### GsDnmt2 shows unusual preference for tRNA<sup>Glu</sup> over tRNA<sup>Asp</sup>

After the initial observation that *G. sulfurreducens* Dnmt2 showed lower methylation of its *Geobacter*-encoded tRNA<sup>Asp</sup> than of tRNA<sup>Asp</sup> from *Drosophila* or *M. musculus*, the question arose whether any other *Geobacter* tRNA might be more preferred as a substrate. To this end, *Geobacter* tRNAs were inspected for the presence of a cytosine at position 38 of the tRNA sequence. Using the UCSC Genomic tRNA Data Base (<http://gtrnadb.ucsc.edu/>) (29) we identified tRNA<sup>His</sup> as well as two isodecoders of each tRNA<sup>Ala</sup>, tRNA<sup>Glu</sup> and tRNA<sup>Val</sup> as Dnmt2 candidate substrates in *Geobacter*, in addition to tRNA<sup>Asp</sup>. Among them, both isodecoders of tRNA<sup>Glu</sup> were selected for methylation analysis, because they show high similarity to tRNA<sup>Asp</sup>, as well as tRNA<sup>His</sup> and one of the tRNA<sup>Val</sup> and tRNA<sup>Ala</sup> sequences (Supplementary Table S1). All of these tRNAs were synthesized by *in vitro* transcription and assayed for methylation by GsDnmt2. Our results showed that tRNA<sup>Asp</sup> was weakly methylated as stated above (Figure 2), but the initial reaction rate for methylation of tRNA<sup>Glu</sup> (tRNA32) was 10-fold higher than methylation of tRNA<sup>Asp</sup>. The methylation of the second isodecoder of tRNA<sup>Glu-2</sup> (tRNA20) was intermediate between tRNA<sup>Asp</sup> and the other tRNA<sup>Glu</sup> (Figure 2) and methylation of other tRNAs was not detectable (data not shown). These results motivated us to continue our study using the tRNA<sup>Glu</sup> (tRNA32) isodecoder. A mutant tRNA<sup>Glu</sup> carrying a C38U mutation was prepared and we observed that the mutation caused a complete loss of methylation, indicating that the methylation in tRNA<sup>Glu</sup> happens only at position C38 (Figure 2). In addition, the C74A mutation in GsDnmt2 led to loss of methylation on tRNA<sup>Glu</sup> (Figure 2). The preferential methylation of Gs-tRNA<sup>Glu</sup> was also confirmed using a gel-based methylation assay (10) in which the methylated tRNA is directly observed (Supplementary Figure S5).

To test if the substrate preferences of human and *Geobacter* Dnmt2 were based on preferential binding of one type of tRNA, we determined their equilibrium constants of binding to Gs-tRNA<sup>Glu</sup> and Gs-tRNA<sup>Asp</sup> (Supplementary Figure S6). Our data show that the human enzyme binds tRNA about four to five times stronger than the *Geobac-*



**Figure 4.** Dnmt2 enzymes from various species prefer methylation of *G. sulfurreducens* tRNA<sup>Asp</sup>. (A) Examples of methylation kinetics are shown for wild-type Dnmt2 enzymes from *S. pombe* (Pmt1), *D. discoideum* (DnmA), *D. melanogaster* and *H. sapiens* using *in vitro* transcribed Gs-tRNA<sup>Asp</sup> and Gs-tRNA<sup>Glu</sup> and their respective C38U variants. (B) Average methylation rates and SEM based on two repeats of the experiments.

*ter* enzyme, which is in agreement with its general higher enzymatic activity (compare Figures 2B and 4B). However, the binding of the two different substrates was similar in both cases, indicating that ground-state tRNA binding does not contribute to specificity of tRNA methylation by Dnmt2. A similar observation has been made previously in a study aiming to identify amino acid residues involved in tRNA binding and recognition of human DNMT2 (23). We conclude that GsDnmt2 shows a striking change in substrate specificity when compared with other Dnmt2 enzymes, which is not based on tRNA-binding preferences.

#### Cellular methylation of tRNAs in *G. sulfurreducens*

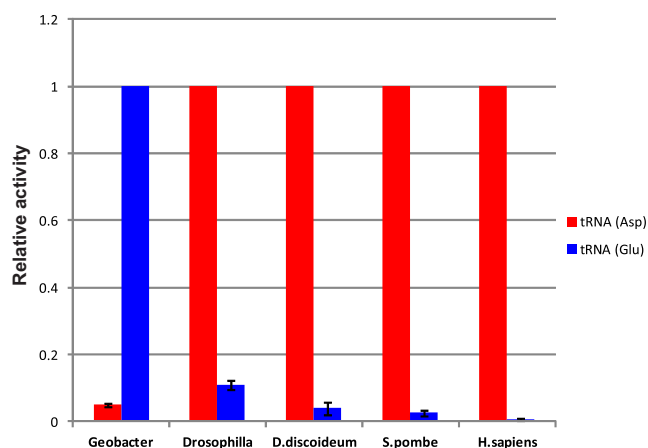
As described above, we have found that GsDnmt2 has a different specificity than other Dnmt2 enzymes *in vitro*. Therefore, the cellular methylation patterns of tRNAs in *G. sulfurreducens* were investigated. For this analysis, total RNA was isolated from wild-type *G. sulfurreducens* and the cytosine methylation investigated by bisulfite conversion, reverse transcription and cloning of the converted tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup> and tRNA<sup>Val</sup> followed by sequencing of individual clones. As shown in Figure 3, the methylation at the C38 position of tRNA<sup>Glu</sup> in wild-type *G. sulfurreducens* was 22%. The tRNA<sup>Glu-2</sup> isodecoder was methylated by 36% at the same site. In contrast, signals for tRNA<sup>Asp</sup> and tRNA<sup>Val</sup> C38 methylation were at background levels of about 3%. Hence, both tRNA<sup>Glu</sup> isodecoders were efficiently methylated *in vitro* and they are also methylated in cells, while tRNA<sup>Asp</sup>, which showed the weakest *in vitro* methylation among all substrates tested, was not methylated in cells.

We conclude that cellular tRNA methylation at C38 mirrors the *in vitro* specificity GsDnmt2. The same observation has been made with human DNMT2. In human cells tRNA<sup>Asp</sup> but not tRNA<sup>Glu</sup> is methylated at C38 (34,35), which is in agreement with the *in vitro* specificity of the human DNMT2 enzyme. The incomplete methylation of the tRNA<sup>Glu</sup> in *Geobacter* is similar to what has been observed for tRNA<sup>Asp</sup> in other species by RNA bisulfite (16,32,34). It is possible that the reverse transcription step of the RNA bisulfite method is more efficient with unmodified, immature tRNAs, which may influence the results.

#### Comparison of methylation specificities of Dnmt2 homologs from various species

We concluded in the last paragraphs that GsDnmt2 strongly prefers to methylate Gs-tRNA<sup>Glu</sup> not Gs-tRNA<sup>Asp</sup> both *in vitro* and *in vivo*. However, as shown above, tRNA<sup>Asp</sup> from other species like *D. melanogaster* and *M. musculus* were good GsDnmt2 substrates. This observation suggested that there may be changes in the *Geobacter* tRNAs that increase methylation of Gs-tRNA<sup>Glu</sup> but reduce the reaction with Gs-tRNA<sup>Asp</sup>. Hence, the specificities of Dnmt2 enzymes from other species were investigated for the methylation of *Geobacter* tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> also using the C38U mutant tRNA versions as controls (Figure 4). Dnmt2 enzymes from different species that represent key eukaryotic phyla were expressed and purified (Supplementary Figure S3) and their methylation activities were analyzed. The assays were done in parallel for a specific enzyme with all tRNA substrates. The results showed that human DNMT2,





**Figure 5.** Comparison of the specificities of Dnmt2 homologs from different species for methylation of *Geobacter* tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup>. Data are replotted from Figures 2B and 4B and the relative activities of the Asp/Glu pairs were always normalized to the more active substrate for a better comparison. Error bars were based on normalized individual experiments and correspond to the SEM of two to three repeats.

*S. pombe* Pmt1 and *D. discoideum* DnmA, all preferred the Gs-tRNA<sup>Asp</sup> substrate (Figure 4). *D. melanogaster* Dnmt2 also showed some activity on Gs-tRNA<sup>Glu</sup>, but it still had a clear preference for Gs-tRNA<sup>Asp</sup> (Figure 4). The C38U mutations completely abrogated the methylation activity for all the enzymes, confirming that methylation occurred at position C38. Although the results with the other Dnmt2 enzymes cannot be directly translated to their biological role, because the methylation was tested on *G. sulfurreducens* tRNAs, our data clearly demonstrate that the change in the biological target observed with GsDnmt2 is specific for this enzyme among all Dnmt2 enzymes tested here (Figure 5). These results show that the swap in specificity of GsDnmt2 from Gs-tRNA<sup>Asp</sup> to Gs-tRNA<sup>Glu</sup> must be due to changes within the GsDnmt2 protein and the Gs-tRNAs which affect the interaction of GsDnmt2 with *Geobacter*-encoded tRNAs. However, these alterations are only efficient when combined, because GsDnmt2 still methylates tRNA<sup>Asp</sup> from other species and Dnmt2 enzymes from other species prefer methylating tRNA<sup>Asp</sup> from *Geobacter*.

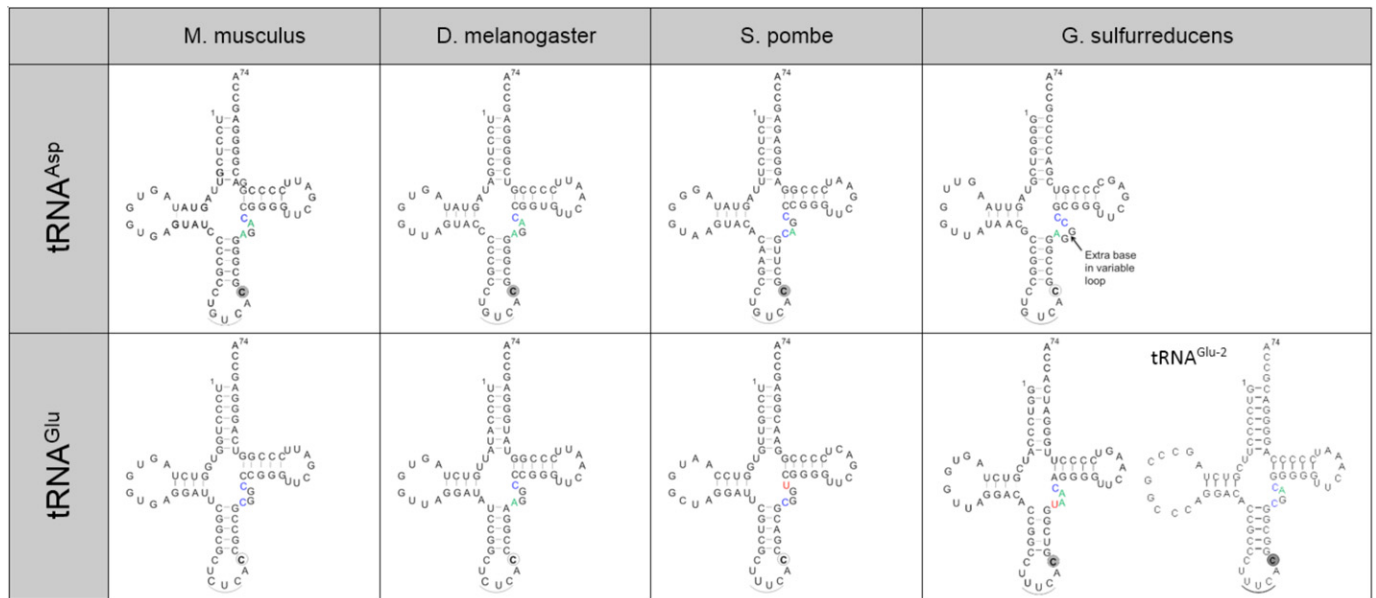
### The variable loop functions as a sequence determinant in the tRNA recognition of Dnmt2

To investigate the molecular reason for the inversion of the substrate preference of the GsDnmt2 enzyme with its matching *Geobacter* tRNAs, we compared the secondary structures of tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> from several species and noticed that Gs-tRNA<sup>Asp</sup> has a longer variable loop with an extra guanine (Figure 6). Furthermore, the variable loops of all preferred Dnmt2 substrates (namely tRNA<sup>Asp</sup> from all species but *Geobacter* and Gs-tRNA<sup>Glu</sup>) have at least one A at position 45 or 46 of the variable loop, while all the inactive counterparts contain a GG dinucleotide. Based on this, we speculated that the variable loop may be a specificity determinant of GsDnmt2. To test this hypothesis, the variable loop of murine tRNA<sup>Asp</sup> was swapped with that of murine tRNA<sup>Glu</sup> by an exchange of two nucleotides (AGAC

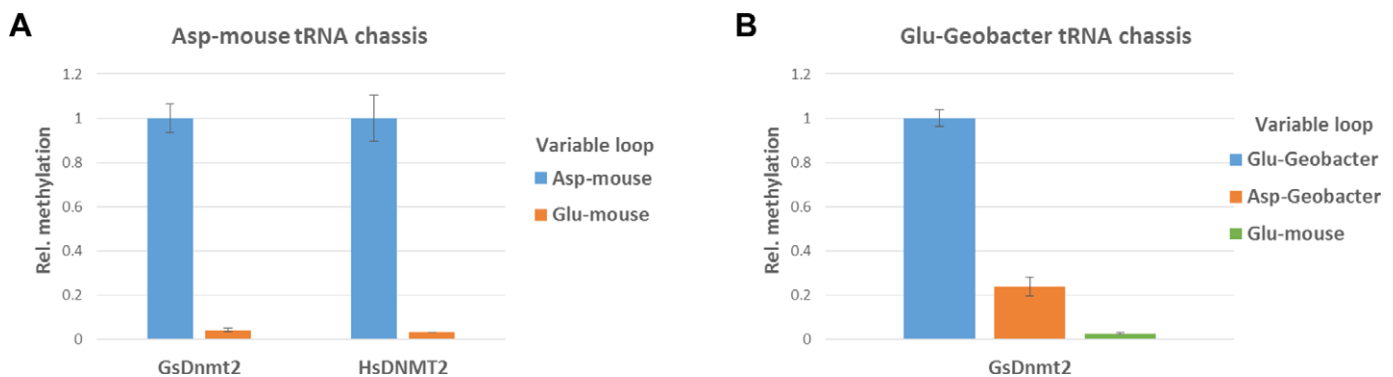
to CGGC) which introduced the GG dinucleotide sequence into tRNA<sup>Asp</sup>. The mutant tRNA<sup>Asp</sup> was synthesized by *in vitro* transcription. Equal amounts of the wild-type and mutant tRNAs were used in methylation reactions with GsDnmt2 (Supplementary Figure S4). Strikingly, the alteration of these two bases in the variable loop of murine tRNA<sup>Asp</sup> led to a strong decrease in the methylation rate (Figure 7A). This result supports the hypothesis of an involvement of the variable loop in the enzyme-substrate recognition of GsDnmt2. To further extend this finding, methylation reactions were performed with the same substrates using the human DNMT2 enzyme. Here, also, a similar reduction of activity was observed with the mutant tRNA<sup>Asp</sup> compared to the wild type tRNA (Figure 7A), indicating that the variable loop is also important for tRNA<sup>Asp</sup> recognition by human DNMT2. Next, we investigated if the variable loop of Gs-tRNA<sup>Glu</sup> also has a role in recognition. As shown in Figure 7B, replacement of the original Gs-tRNA<sup>Glu</sup> variable loop by either Gs-tRNA<sup>Asp</sup> or murine tRNA<sup>Glu</sup> resulted in a strong reduction of its methylation by GsDnmt2. These data indicate that the GG dinucleotide in the variable loop functions as a universal anti-determinant for methylation by Dnmt2. However, replacing the variable loop of murine tRNA<sup>Glu</sup> with the corresponding loop from murine tRNA<sup>Asp</sup> did not result in a significant increase of its methylation by human DNMT2 (data not shown), indicating that the variable loop is not the only recognition determinant of Dnmt2.

### DISCUSSION

Dnmt2 enzymes are conserved in most eukaryotic species (6,9) and methylate tRNA<sup>Asp</sup> with high activity. Here, the activity of one of the few known bacterial putative Dnmt2 enzymes was studied, which is found in *Geobacter* strains including *G. sulfurreducens* (GsDnmt2). We show that GsDnmt2 actively methylates tRNA<sup>Asp</sup> from *M. musculus* and *Drosophila* that were used as model substrates in previous work. This result confirms that GsDnmt2 is a member of the Dnmt2 family as expected on the basis of the amino acid alignment. However, the endogenous *Geobacter* tRNA<sup>Asp</sup> was methylated only very inefficiently. Instead, GsDnmt2 methylates *Geobacter* tRNA<sup>Glu</sup> at the corresponding site, indicating a complete swap in its specificity (Figure 5)—a surprising and unexpected result that was confirmed in a methylation analysis of tRNA isolated from *G. sulfurreducens*. There are two isodecoders of tRNA<sup>Glu</sup> in *Geobacter* (tRNA<sup>20</sup>, here called tRNA<sup>Glu-2</sup>, and tRNA<sup>32</sup>, here called tRNA<sup>Glu</sup>), which both were methylated *in vitro* and *in vivo*, although the relative methylation levels of the two isodecoders varied in the two data sets. *In vitro*, tRNA<sup>Glu</sup> was methylated three times faster while in cells the methylation level of tRNA<sup>Glu-2</sup> was 1.6 times higher. There could be several explanations for this difference: (i) tRNA<sup>Glu-2</sup> methylation could be less efficient under *in vitro* conditions, (ii) additional modifications could modulate the tRNA<sup>Glu</sup> or tRNA<sup>Glu-2</sup> methylation *in vivo* or (iii) additional modifications could affect the recovery of methylated tRNA<sup>Glu</sup> and tRNA<sup>Glu-2</sup> differently after bisulfite conversion. However, in all data sets both isodecoders of Gs-tRNA<sup>Glu</sup> were more methylated than Gs-tRNA<sup>Asp</sup>.



**Figure 6.** Clover leaf structure of the tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> from different species. The target C38 is printed in bold and highlighted by a gray circle for the preferred substrate tRNA and by a white circle for the non-preferred one. Bases in the variable loop are colored. The sequences of human tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> are identical to that of murine-tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup>.



**Figure 7.** Methylation of tRNA variable loop mutants by GsDnmt2 and human DNMT2. (A) Methylation of murine-tRNA<sup>Asp</sup> with its original variable loop or with the variable loop of murine-tRNA<sup>Glu</sup> by *Geobacter* Dnmt2 (left pair of bars) or human DNMT2 (right pair of bars). (B) GsDnmt2 methylation of Gs-tRNA<sup>Glu</sup> with its original variable loop or with variable loops from Gs-tRNA<sup>Asp</sup> or murine-tRNA<sup>Glu</sup>. The figure shows averages of the methylation rates normalized to the wild-type tRNA. Error bars indicate SEM derived from two repeats of the experiments.

We have shown here that *Geobacter* Dnmt2 has an ~100-fold swapped specificity for *Geobacter* tRNAs, because it prefers tRNA<sup>Glu</sup> roughly 10-fold over tRNA<sup>Asp</sup>, while all other Dnmt2 enzymes showed an at least 10-fold preference for tRNA<sup>Asp</sup> over tRNA<sup>Glu</sup> (Figure 5). What could be the molecular basis of the unexpected loss of *Geobacter* tRNA<sup>Asp</sup> methylation by GsDnmt2 and the corresponding gain of methylation of tRNA<sup>Glu</sup>? So far, various amino acids involved in tRNA recognition of the human enzyme could be mapped (23), but the sequence elements needed on the tRNA side were largely unknown. Initially, the G34 base at the wobble position of the anticodon, which is modified to mannosylqueosine in eukaryotic cells, was associated with Dnmt2 activity (6). However, the results with *G. sulfurreducens* are not in favor of that model, because Gs-tRNA<sup>Asp</sup> contains the G34, but it is a very weak substrate, while Gs-tRNA<sup>Glu</sup>, which is the preferred substrate,

does not have a G34. By comparing substrate and non-substrate tRNAs of GsDnmt2, it became apparent that all non-substrates contain a GG dinucleotide in the variable loop as a characteristic feature. Indeed, introduction of this sequence into murine tRNA<sup>Asp</sup> and *Geobacter* tRNA<sup>Glu</sup> drastically reduced their methylation by GsDnmt2. We cannot rule out the possibility that the two point exchanges in the variable loop might disrupt tRNA folding. However, the initial discovery that the GG dinucleotide is inhibiting GsDnmt2 was made with natural tRNAs, which are all fully functional in protein biosynthesis, which rules out loss of structure in these cases. Therefore, when taken together, our results strongly suggest that the variable loop functions as an important specificity determinant in GsDnmt2. Interestingly, the exchange in the murine tRNA<sup>Asp</sup> also reduced its methylation by the human DNMT2, indicating that the variable loop is a critical specificity determi-



nant for the tRNA recognition of human DNMT2 as well. However, introduction of the favorable variable loop into murine tRNA<sup>Glu</sup> did not increase its methylation. Hence, our data indicate that Dnmt2 enzymes use further tRNA-specific sequence determinants in addition to the variable loop. These (so far unknown) contacts mediate the preference of the human DNMT2 enzyme for Gs-tRNA<sup>Asp</sup>, despite the presence of the unfavorable variable loop. Furthermore, they prevent methylation of murine tRNA<sup>Glu</sup>, even after introducing a favorable variable loop. The contribution of additional specificity determinants (besides the GG dinucleotide in the variable loop) to tRNA recognition by Dnmt2 enzymes is also supported by the observation that Dnmt2 enzymes from other species still strongly prefer Gs-tRNA<sup>Asp</sup> over Gs-tRNA<sup>Glu</sup>.

The role of the variable loop as a specificity determinant in tRNA recognition by Dnmt2 is not without precedence. Examples for tRNA-interacting enzymes, which use the variable loop for tRNA recognition, include a bacterial tRNA (m7G46) methyltransferase (36) and a prokaryotic tRNA-dependent amidotransferase (37), and the same strategy is also used by several aminoacyl-tRNA-synthetases (38). Recognition of nucleic acids by proteins, in general, is based on direct and indirect readout, i.e. direct contacts of amino acids to nucleobases, which detect the nature of the base, and contacts to the nucleic acid backbone, which detect the structure of the nucleic acid. The GG dinucleotide in the variable loop of the tRNA could interfere with either of these processes. However, the observation that the variable loop of preferred tRNA substrates is only characterized by the absence of the GG dinucleotide but it does not contain a specific nucleotide sequence might argue against direct base contacts. This suggests that an indirect readout mechanism is operational in which the presence of the GG dinucleotide induces a structural change in the tRNA which then prevents its methylation.

The finding that GsDnmt2 methylates tRNA<sup>Asp</sup> from other species (as all other members of the Dnmt2 family do) but not from *Geobacter* provides strong support for the hypothesis that the Gs-Dnmt2 was acquired by horizontal gene transfer, because there is no reason for this enzyme to methylate tRNA<sup>Asp</sup>, which is not a substrate in *Geobacter* itself. In the horizontal gene transfer view, the activity toward tRNA<sup>Asp</sup> can be interpreted as an evolutionary relict. Based on our data, one may speculate that initially a *Dnmt2* gene with tRNA<sup>Asp</sup> preference, but also the ability to methylate tRNA<sup>Glu</sup>, was acquired by *Geobacter*. Precedence for such side activity can be seen in many species including *D. melanogaster*, *S. pombe* and *D. discoideum* (13,15,16). Methylation of the Gs-tRNA<sup>Glu</sup> was favorable, which led to the stable integration of the Dnmt2, whereas methylation of the endogenous tRNA<sup>Asp</sup> was lost in a co-evolution of GsDnmt2 and the Gs-tRNA<sup>Asp</sup>. At present, one can only speculate about the role of Dnmt2 in *Geobacter*. In previous studies, tRNA methylation by Dnmt2 was found to improve the stability of the tRNA (13) or affect tRNA charging (R. Shanmugam *et al.*, in preparation), and prevent tRNA fragmentation (13). Which of these roles, if any, is important for *Geobacter* tRNA<sup>Glu</sup> cannot be said at present. The generation of a Dnmt2 knock-out strain was unsuccessful (M. Akuljar, unpublished information), which may indicate that

*Dnmt2* is an important gene in *G. sulfurreducens*, which is in line with the evolutionary arguments. This would be an interesting finding, since Dnmt2 enzymes are non-essential in other model organisms under normal growth conditions (6,12,13,15,21).

## CONCLUSIONS

The first enzymatic characterization of the specificity of a bacterial Dnmt2 homolog, the enzyme from *G. sulfurreducens*, revealed that it is the only known Dnmt2 enzyme that does not prefer methylation of tRNA<sup>Asp</sup>. Instead, an unexpected swap of specificity of this enzyme was observed toward methylation of *Geobacter* tRNA<sup>Glu</sup>, which was confirmed *in vitro* and *in vivo*. However, GsDnmt2 still methylates tRNA<sup>Asp</sup> from other species, and Dnmt2 enzymes from other species still methylate *Geobacter* tRNA<sup>Asp</sup>. We provide an evolutionary scenario that can explain this interesting finding. We noticed that the *Geobacter* tRNA<sup>Asp</sup> has a modified variable loop, which serves as a specificity determinant and causes the loss of methylation activity. Furthermore, we show that this mechanism could be extended to the human enzyme as well, indicating that work with a bacterial Dnmt2 homolog, finally, has led to important insight into the mechanism of the corresponding human enzyme.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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