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Biochemical characterization of ParI, an orphan C5-DNA methyltransferase from *Psychrobacter arcticus* 273-4

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ABSTRACT

Cytosine-specific DNA methyltransferases are important enzymes in most living organisms. In prokaryotes, most DNA methyltransferases are members of the type II restriction-modification system where they methylate host DNA, thereby protecting it from digestion by the accompanying restriction endonucleases. DNA methyltransferases can also act as solitary enzymes having important roles in controlling gene expression, DNA replication, cell cycle and DNA post-replicative mismatch repair. They have potential applications in biotechnology, such as in labeling of biopolymers, DNA mapping or epigenetic analysis, as well as for general DNA-protein interaction studies.

The *par*I gene from the psychrophilic bacterium *Psychrobacter arcticus* 273–4 encodes a cytosine-specific DNA methyltransferase. In this work, recombinant ParI was expressed and purified in fusion to either an N-terminal hexahistidine affinity tag, or a maltose binding protein following the hexahistidine affinity tag, for solubility improvement. After removal of the fusion partners, recombinant ParI was found to be monomeric by size exclusion chromatography, with its molecular mass estimated to be 54 kDa. The apparent melting temperature of the protein was 53 °C with no detectable secondary structures above 65 °C. Both recombinant and native ParI showed methyltransferase activity *in vivo*. In addition, MBP- and His-tagged ParI also demonstrated *in vitro* activity. Although the overall structure of ParI exhibits high thermal stability, the loss of *in vitro* activity upon removal of solubility tags or purification from the cellular milieu indicates that the catalytically active form is more labile. Horizontal gene transfer may explain the acquisition of a protein-encoding gene that does not display common cold-adapted features.

1. Introduction

The methylated DNA bases N4-methylcytosine, N6-methyladenine and C5-methylcytosine are considered additional bases of the genetic code that carry epigenetic information not encoded in the DNA sequence itself (reviewed in Refs. [1] and [2]). Generally, DNA methylation has an important role in protein-DNA interaction by either enhancing or disrupting binding of proteins to DNA. The enzymes responsible for these modifications are DNA methyltransferases (DNA-MTases), which catalyze the transfer of a methyl group from S-adenosyl-*L*-methionine (SAM) to the three above-mentioned positions in DNA [3]. In contrast to the role of eukaryotic DNA-MTases, most prokaryotic DNA-MTases are members of a host protection system, the restriction-modification (RM) system [4,5]. The main function of DNA-MTases in the RM system is methylation of host DNA which confers protection from digestion by restriction endonucleases (REases) that recognize the same specific DNA sequence [6]. Some DNA-MTases are not accompanied by an REase and are so-called orphan MTases [7]. The roles of orphan MTases have been proposed to be within gene regulation, DNA replication, cell cycle and directing post-replicative mismatch repair on newly synthesized DNA strands by *de novo* methylation [2,8–12].

In contrast to eukaryotic DNA 5-cytosine methyltransferases (C5-DNA-MTases), being multi-domain proteins and complexes, the prokaryotic enzymes are single-domain proteins [13]. When three-dimensional structures of C5-DNA-MTases are compared, both prokary-

Abbreviations: TEV, tobacco etch virus; DSC, differential scanning calorimetry; CD, circular dichroism; DNA-MTase, DNA methyltransferase; C5-DNA-MTase, DNA 5-cytosine methyltransferase; MBP, maltose binding protein.

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otic and eukaryotic catalytic DNA-MTase domains are similar in structural organization. The catalytic domains are organized into a large and a small sub-domain separated by a marked cleft [14]. Despite their structural homology, the sequence similarity among C5-DNA-MTases is low with the exception of ten conserved motifs, named with roman numerals I-X [3]. In addition to these motifs, all C5-DNA-MTases possess a variable region; the Target Recognition Domain (TRD), which is involved in sequence recognition [3,9]. Most of the conserved motifs are located in the large sub-domain, while the TRD comprises most of the small sub-domain. The best conserved motifs (I, IV, VI, VIII, IX and X) are either structural (motif IX), or involved in SAM binding (motifs I and X), DNA binding (motifs VI, VIII and TRD) or catalysis (motif IV) [3,14–16].

Psychrobacter arcticus 273–4 is a gram-negative bacterium discovered in 20–30 thousand year old permafrost soil in Kolyma, Siberia [17]. *P. arcticus* 273-4 has been genome sequenced [18], grows at temperatures from −10 to 28 °C and has generation time of 3.5 days at temperatures below zero [17–20]. At the protein level, *P. arcticus* 273–4 possesses many common features for psychrophilic bacteria, such as reduced use of proline, arginine and acidic amino acids, an increased lysine content, as well as encoding several cold shock proteins [18–21].

In the present study, a C5-DNA-MTase from *P. arcticus* 273–4, ParI, was characterized on the basis of its potential to possess features relevant for biotechnological applications, such as labeling of biopolymers, DNA mapping or epigenetic analysis [22–24].

2. Materials and methods

2.1. Bioinformatic analyses

A multiple protein sequence alignment where ParI was compared to bacterial C5-DNA-MTase homologs [HhaI from *Haemophilus parahaemolyticus* (GI: 127455**);** M. SssI from *Spiroplasma* sp. (GI: 417325); AbaI from *Acinetobacter baumannii* UH5107 (GI: 446969424)] and the catalytic domain of human DNMT1 (GI: 12231019) was made using the T-Coffee web server [25]. The sequence alignment was rendered with the ESPript server [26]. The sequence alignment was annotated with secondary structure of HhaI (PDB ID: 1MHT) and ParI secondary structure predictions from the PsiPred server [27]. The genomic context of *ParI* in *P. arcticus* 273–4 was analysed by the PHAST server [28]. Promoter prediction was performed with **BPROM** [29], while the Rho-independent terminators were predicted by **ARNold** [30,31].

2.2. Cloning, expression and purification

The gene encoding ParI, *parI* [GenBank ID: 71038525] was amplified from *P. arcticus* 273–4 (DSMZ) with primers synthesized by Sigma-Aldrich and Phusion polymerase (NEB) in a PCR reaction following the protocol for Phusion polymerase. The amplified gene, including a primer-encoded TEV-cleavage site, was cloned into either the pDest17 vector which encodes a His tag (resulting in pHis-ParI), or the pHMGWA vector encoding an N-terminal hexahistidine (His) tag and maltose binding protein (MBP). Both constructs were cloned using the Gateway technology (Thermo Fisher Scientific), according to the protocol provided by the manufacturer [32]. All primers used are listed in Tables S–2. Newly cloned constructs were transformed by the heat-shock method into the McrBC-negative *E. coli* T7 Express strain (NEB) for recombinant expression and purification (genotype *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--*TetS*)2 [dcm] R(zgb-210::Tn10--*TetS*) endA1 Δ(mcrC- mrr)114::IS10*). The protein was expressed in 1L cultures of Lysogeny broth (LB) media and induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, VWR) at a final concentration of 0.5 mM. Cells for both His-ParI and His-MBP-tagged ParI were harvested after 7 h expression at 20 °C, by centrifugation at 7500 × *g* for 30 min at room temperature and resuspended in lysis buffer (50 mM Tris pH 8.0, 750 mM NaCl, 5% (v/v) glycerol, 10 mM $MgCl_2$) supplemented with 1x Complete protease inhibitor cocktail (Roche). The cells were disrupted by a cell disruptor (Constant Systems, Ltd.) using a pressure of 1.35 kbar in four cycles. The lysate was cleared by centrifugation at 20000 × *g* for 30 min at 4 °C after which it was incubated with HL/SAN DNase (ArticZymes) for 1 h at 4 °C. Affinity purification of recombinant His-ParI or His-MBP-ParI was carried out on a 5 ml HisTrap HP column (GE Healthcare) equilibrated with buffer A (50 mM Tris pH 8.0, 750 mM NaCl, 5% (v/v) glycerol and 10 mM imidazole) using an ÄKTA purifier (GE Healthcare). The bound protein was eluted across a gradient of 0–100% buffer B (50 mM Tris pH 8.0, 750 mM NaCl, 5% (v/v) glycerol and 500 mM imidazole). The purity of the protein was evaluated by SDS-PAGE. In the case of His-MBP-tagged ParI, the removal of the fusion partner was carried out in buffer C (50 mM Tris pH 8.0, 200 mM NaCl, 5% (v/v) Glycerol, 1 mM DTT and 0.5 mM EDTA) supplemented with TEV protease in a 1:10 mg/mg ratio to ParI and incubated overnight at 4 °C. Buffer exchange was done using a HiPrep 26/60 desalting column (GE Healthcare). The recombinant ParI protein was recovered from the flow-through after a second step of HisTrap affinity purification, while the His-MBP portion remained bound to the column. To determine the oligomeric state of the protein, untagged ParI was separated on a HiLoad 16/60 Superdex 200 prep grade gel filtration chromatography column (GE Healthcare) in buffer C. The protein concentration was determined by measuring the absorbance at 280 nm using a Nanodrop spectrophotometer (NanoDrop Technologies). The theoretical extinction coefficient for the protein is 63995 $M^{-1}cm^{-1}$ as calculated by the ProtParam tool.[1] Pure protein was concentrated by centrifugation through 10 kDa MWCO Amicon Ultra Centrifugal filters (Merck Millipore). The identity of recombinant protein was confirmed by MS/MS by the Proteomics facility (UiT, The Arctic University of Norway).

2.3. Thermofluor assay

To assess the stability of ParI, a fluorescence-based thermal shift (Thermofluor) assay was used [33]. A buffer screen consisting of 24 different buffers at various pHs was performed. (Bicine, pH 8; Bicine, pH 9; Hepes, pH 7; Hepes, pH 7.5; Hepes, pH 8; Imidazole, pH 8; MES, pH 6; MES, pH 6.2; MES, pH 6.5; Potassium phosphate, pH 5; Potassium phosphate, pH 6; Potassium phosphate, pH 7; Sodium acetate, pH 4.5; Sodium acetate, pH 5; Sodium cacodylate, pH 6.5; Sodium citrate, pH 4.7; Sodium citrate, pH 5.5; Sodium phosphate, pH 5.5; Sodium phosphate, pH 6.5; Sodium phosphate, pH 7.5; Tris, pH 7.5; Tris, pH 8; Tris, pH 8.5). The final concentration of protein in the reaction was 0.5 mg/ml. SYPRO® Orange Protein Gel Stain (Sigma-Aldrich) was diluted 1:75 from the starting concentration. The assay was performed in a volume of 25 µl and was run in a MiniOpticon real-time PCR system (BioRad) in a temperature range from 5 °C to 95 °C with 1 °C increment every 3 s.

2.4. Differential scanning calorimetry

Thermal denaturation curves were recorded at temperatures between 5 °C and 95 °C using a scan rate of 1 °C/min in an N-DSC III calorimeter (Calorimetry sciences corp.). ParI was dialyzed against DSC-buffer (50 mM HEPES pH 8.0, 200 mM NaCl) and used at a final

[1] Expasy server: http://web.expasy.org/protparam/.

concentration of 1.4 mg/ml. The DSC-buffer from dialysis was used as a reference.

2.5. Circular dichroism

ParI was dialyzed overnight at 4 °C against CD-buffer (10 mM Tris pH 8.0 and 100 mM NaF). The samples were filtered through a 0.45 μm pore size filter (Spin X Costar) to remove precipitate and diluted to a final concentration of 0.15 mg/ml. SAM was added at a final concentration of 200 μM to either protein or buffer directly before measurement. Data was collected on a J-810 CD spectrophotometer (Jasco) using a 1 mm path length cuvette and the following settings: sensitivity 100 mdeg, datapitch 0.5 nm, scan speed 50 nm/min, response 2.0 s, bandwidth 1 nm, accumulation three scans, units CD mdeg. Three scans were recorded and averaged. Three scans of buffer were also recorded in absence or presence of SAM. The measurements were done at 15 °C, 35 °C and 65 °C. The measurement at 15 °C was done in the presence and absence of SAM, while measurements at 35 °C and 65 °C were performed without SAM. In the data analysis, each set of spectra corresponding to each condition was analysed with two different programs, using the SP170 reference set [34–41]. Data analysis was conducted through the Dichroweb server [39].

2.6. In vivo MTase genome assay

To investigate His-ParI activity, *P. arcticus* was cultivated in LB media at 15 °C for 3 days while the methylase-deficient control, a closely related mesophilic *Psychrobacter* sp. PRwf-1, which lacks a C5-DNA-MTase, was cultivated at 37 °C overnight. Recombinant ParI activity was determined by transforming 50 μl cultures of McrBC-negative *E. coli* T7 Express strain (NEB) with the pHis-ParI construct. Cells were grown until late log phase (OD 0.7–0.9) before induction of recombinant protein with IPTG, and further cultivation at 20 °C for 7 h. Untransformed T7 Express cells were treated in the same manner and served as a negative control. Genomic DNA from all bacteria was extracted using the GenElute Gel extraction Kit (Sigma Aldrich), and treated with McrBC endonuclease (NEB) in 1x NEB2 buffer (NEB) supplemented with 200 μg/ml BSA and 1 mM GTP, and incubated at 37 °C for 1 h. The reaction mix was separated by 1% agarose gel electrophoresis and the DNA was post-stained with RedSafe (iNtRON Biotechnology). Digestion by McrBC relative to the negative controls was taken as an indication of DNA methylation by ParI. As a positive control, pUC19 vector methylated with M. SssI methyltransferase (New England Biolabs) was used.

In a second *in vivo* assay, adapted from Ref. [42], the pHis-ParI construct was transformed into two different *E. coli* expression strains, BL21Star (DE3) (Life Technologies) and the McrBC-negative T7 Express (NEB). Cells were transformed as previously described and plated on LB plates containing 100 μg/ml ampicillin and 0.2 mM IPTG. The plates were incubated at 37 °C overnight.

2.7. In vitro methyltransferase activity assay with radiolabelled SAM (3H-SAM)

The reactions were performed with 25 ng DNA (pUC19 vector), 1 mg/ml of either purified proteins (His-MBP-ParI, His-ParI and ParI) or clarified lysates (His-MBP-ParI (lysate) and His-ParI (lysate)) and 14 μM 3H-SAM in methyltransferase buffer (NEB2 buffer: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT), adjusted to a total volume of 20 μl with nuclease-free H_2O. The samples were incubated at RT for 120 min. After incubation, reactions were spotted onto Filtermat A, 24 Well (PerkinElmer). Filters were then washed three times with 50% TCA and once with 100% Ethanol. The filters were dried and analysed by liquid scintillation counting (MicroBeta2, 2450 microplate counter, PerkinElmer) using scintillation liquid (Ultima Gold XR, PerkinElmer); reading 1 min per well. The same procedure was followed for M. SssI which was used as a positive control in the experiments, although not included in the results as its molar concentration was unknown. All reactions were blank-corrected against the same setup with no protein added. 3H-SAM was purchased from PerkinElmer. M. SssI and NEB2 buffer were purchased from New England Biolabs. The presented results are triplicates of each sample. Differences in molar protein concentration between the constructs were adjusted for.

3. Results

3.1. Bioinformatic analyses of the parI gene

To investigate the genetic context of ParI, analysis of the RM complement of the *P. arcticus* 273-4 genome and prediction of prophage-containing regions were performed. Through queries in the REBASE database of restriction enzymes [5], several putative RM systems in the *P. arcticus* 273-4 genome were identified, including one complete, but uncharacterized type I RM system, as well as two solitary RM enzymes homologous to type II DpnI-like REases and C5-DNA-MTases (ParI), respectively (Table 1). However, no interaction partners that would constitute functional type II RM systems were identified for either of these two solitary enzymes.

Table 1
***P. arcticus* 273–4 RM systems identified using the REBASE database.** Enzymes are categorized according to the type of restriction modification they belong to (Type I, II or IV). Predicted protein function and name are listed as given in REBASE; genomic location and locus tag are listed as given in the *P. arcticus* 273-4 genome. ParI is termed M.ParAORF980P in REBASE.

Type	Function[a]	Name[b]	Genomic location (bp)	Locus tag
I	M	M.ParAORF415AP	517547–517894	Psyc_0413
	M	M.ParAORF415BP	518942–520957	Psyc_0415
	S	S.ParAORF415P	520957–522330	Psyc_0416
	R	ParAORF415P	528255–531563	Psyc_0424
	R	ParAORF723P	862503–863966	Psyc_0724
	M	M.ParAORF877P	1044227–1045816	Psyc_0877
	S	S.ParAORF877P	1045806–1047080	Psyc_0878
	R	ParAORF877P	1049066–1052248	Psyc_0881
II	R	ParAORF142P	169265–170029	Psyc_0143
	M	M.ParAORF401P	500671–502233	Psyc_0401
	R	ParAORF401P	502506–503840	Psyc_0402
	M	M.ParAORF980P	1175124–1176590	Psyc_0980
IV	R	ParAMcrBP	266672–268168	Psyc_0226

[a] Predicted protein functions are listed as Restriction (R), Modification (M) or Specificity (S).
[b] As given in REBASE.

Further investigation of the *P. arcticus* 273-4 genome in REBASE indicated that *par*I (locus tag Psyc_0980) is part of an operon consisting of four genes from locus tag Psyc_0981 to locus tag Psyc_0978, the rest of which are designated as hypothetical proteins (Fig. 1). The assignment of these genes as an operon is further supported by the prediction of a promoter in front of the gene from locus tag Psyc_0981 and two Rho-independent terminators after the gene with locus tag Psyc_0978 (Fig. 1) which would be capable of regulating transcription of the gene cluster. The genome of *P. arcticus* contains three prophage regions, including one complete temperate phage and two regions where phage genes are clustered (Tables S–1). Although the *par*I gene was not found to be part of any of these prophage regions, the flanking genes were identified as being of phage origin. Previously, two almost complete phage genomes in the *P. arcticus* genome were identified [18], the second of which is located between bp 1177957 and 1223710 and is most similar to the genome of a 45.7 kb MU phage. This region is directly downstream of *par*I and encodes the genes with locus tags Psyc_0981 and Psyc_0982.

Additional BLAST searches of the individual genes from the cluster showed that the gene with locus tag Psyc_0979 has strong nucleotide sequence similarity (82%) to a gene from a temperate bacteriophage Psymv2 isolated from *Psychrobacter* sp. MV2 which indicates that these genes may have been horizontally transferred and are of phage origin, although they are no longer part of an intact temperate phage [43].

3.2. Sequence homology of ParI to other methyltransferases

A multiple sequence alignment of the *P. arcticus* 273-4 ParI protein sequence with other C5-DNA-MTase sequences identified most of the conserved motifs specific for these enzymes (Fig. 2). In motif IV, the PCQ catalytic sequence, which is generally highly conserved among C5-DNA-MTases [14], is replaced by DCK in ParI. BLAST searches revealed that ParI shared the highest sequence similarity (64%) to an uncharacterized C5-DNA-MTase from *Acinetobacter baumannii* UH5107, which also possesses the same PCQ-to-DCK substitution in motif IV. This ParI homologue in *A. baumannii* is located in a region that is recognized as a phage region by PHAST. Both these bacteria belong to the same family, *Moraxellaceae,* which may explain the high sequence similarity [44].

3.3. Expression and purification of recombinant ParI

The *mcr* locus has previously been shown to reduce recovery of methylated sequences from an organism containing methylated cytosines [45]. DNA methylated by active recombinant ParI would be a substrate for digestion by the McrBC gene product. For this reason, an McrBC-negative *E. coli* strain was selected for ParI expression.

Recombinant N-terminally tagged ParI (His-ParI) was successfully produced and purified in small amounts (Fig. 3A), however the yield was extremely low relative to native *E. coli* proteins meaning that large culture volumes (up to 8L) were necessary to obtain mg quantities of pure protein. Furthermore, His-ParI appeared to be very unstable and was prone to aggregation during up-concentration to >1 mg/ml. Neither longer cultivation times (overnight) nor use of autoinduction media improved yields, both resulting in un-detectable His-ParI expression (data not shown). To increase protein yields to quantities suitable for biophysical characterization, ParI was produced in fusion to an N-terminal His-tag and maltose binding protein (His-MBP), the latter of which is known to promote solubility (Fig. 3B) [46,47]. The MBP fusion partner was successfully removed by treatment with tobacco etch virus (TEV) protease (Fig. 3B), although there was a substantial loss of ParI during subsequent chromatographic purification steps after MBP-tag removal. This method typically yielded 2.5 mg of untagged ParI per L of expression culture. Size exclusion chromatography coincided with the theoretical protein molecular weight of 54 kDa indicating the protein is a monomer in solution (Fig. 4). No DNA contamination was observed in the purified protein based on its absorption at 260 nm.

3.4. Thermal stability of ParI

As initial experiments showed that there was a substantial loss of soluble protein during purification, thermal denaturation assays were conducted to assess the general stability of ParI in a variety of buffer conditions. Among the tested buffers, HEPES pH 8.0 was determined to be the most stabilizing. ParI unfolding in HEPES at pH 8.0, monitored using the Thermofluor assay [33], indicates a melting temperature of 53 °C. In this experimental condition the melting curve was broad, stretching over a temperature range of 45–65 °C, which is typically observed in partially unfolded proteins [33].

The thermal denaturation temperature was supported by differential scanning calorimetry (DSC) measurements of ParI, which indicated that this construct unfolds in a single transition event with a melting temperature of 54 °C, ΔH of 82.81 kcal/mol and ΔS of 0.2411 kcal/(mol K) (Fig. 5A). The range of the unfolding transition measured by DSC was 49–59 °C, which is consistent with the Thermofluor results. Thermal denaturation of ParI was found to be irreversible, and after the experiment, precipitated protein was observed in the calorimetric cell.

To further investigate the integrity of ParI, we recorded circular dichroism (CD) spectra (Fig. 5B) [48]. Our analyses indicate that both at 15 °C and 35 °C ParI comprises 20% α-helix 30% β-strand and 12% turns, while the remaining 35–40% of the structure are disordered loops and turns. This is consistent with the secondary structure content predicted by the PsiPred server (Fig. 2). The CD spectrum of ParI at 65 °C was typical for unfolded protein; no secondary structures were observed, which correlates with the Thermofluor and DSC measurements (Fig. 5B). Furthermore, no change in secondary structure was observed in CD measurements at 15 °C in the presence of SAM, indicating that binding of SAM does not induce major structural changes in ParI, or possibly that the recombinant protein was defective in SAM binding.

Thus, three independent biophysical methods indicate that ParI is in a fully folded state with intact secondary and tertiary structure at temperatures below 35 °C, and that it denatures at temperatures above 53 °C.

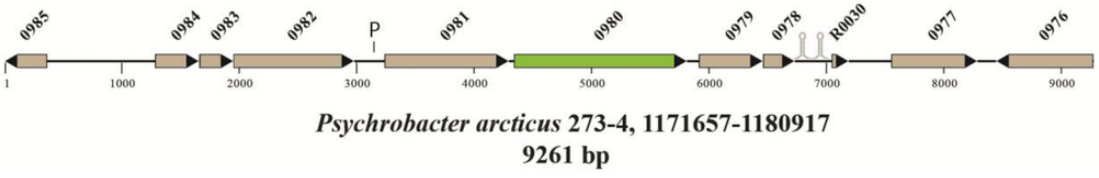

Fig. 1. Gene arrangement surrounding the ParI-encoding gene 0980 of *P. arcticus* 273–4. The cartoon depicts the genome region 1171657–1180917 of *P. arcticus* 273–4, which is 9261 bp long. A promoter (P) was predicted upstream of gene 0981 (green) and two Rho-independent terminators (hairpins) are predicted downstream of gene 0978. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

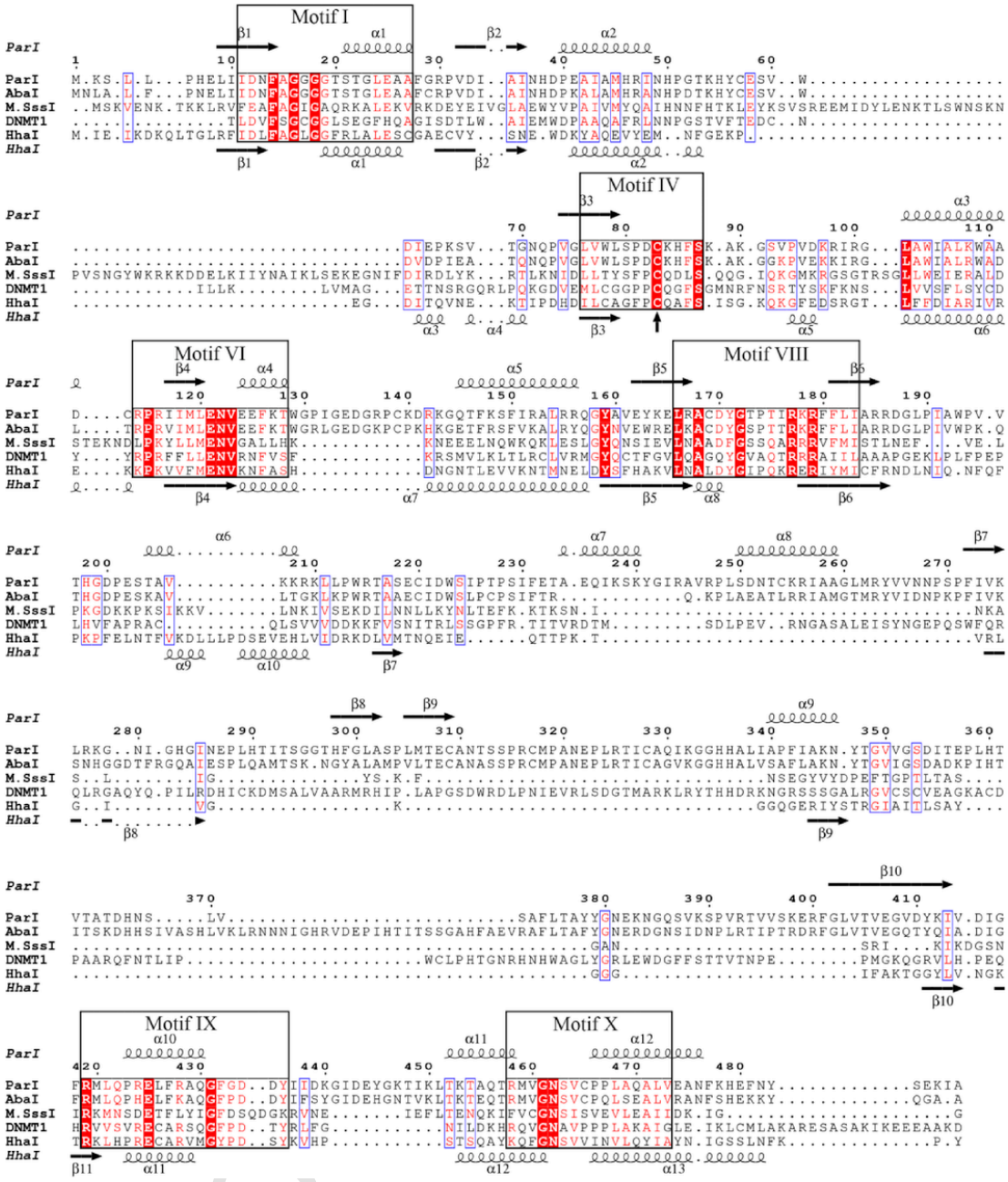

Fig. 2. Multiple sequence alignment of selected bacterial C5-DNA-MTases and human DNMT1. The best conserved motifs between MTases are marked with roman numbers and boxed. Motif IX is structural, motifs VI and VIII are involved in DNA binding and motifs I and X are involved in SAM binding. The catalytic cysteine in motif IV is marked with a black vertical arrow. The top secondary structure elements for ParI, shown as arrows for β strands and spirals for α helices, are predicted using the PsiPred server [27]. Identical residues are marked by red backgrounds, whereas similar residues are given as red letters. The bottom secondary structure elements were rendered from HhaI [PDB: 1MHT]. HhaI, C5-DNA-MTase from *Haemophilus parahaemolyticus;* ParI, C5-DNA-MTase from *Psychrobacter arcticus* 273–4; M. SssI, C5-DNA-MTase from *Spiroplasma* sp.; AbaI, C5-DNA-MTase from *Acinetobacter baumannii* UH5107; DNMT1, from *Homo sapiens* (showing sequence regions encoding the catalytic domain). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Both recombinant and native ParI possess methyltransferase activity in vivo

To determine whether the recombinant His-ParI was active, we used an *in vivo* assay where we screened for McrBC sensitivity of genomic DNA in the presence or absence of ParI. Briefly, genomic DNA was extracted from cultures of MTase-deficient *E. coli* that had been transformed with a plasmid encoding the gene for His-ParI. Genomic DNA extracted from these His-ParI-expressing *E. coli* was digested upon treatment with McrBC, whereas genomic DNA from the ParI-deficient control cells (transformed with empty vector) remained intact (Fig. 6). As McrBC digests both hemi- and fully-methylated DNA, this result strongly suggests that recombinant His-ParI exhibits methyltransferase activity *in vivo*.

Transformation of the plasmid encoding His-tagged ParI into regular *E. coli* BL21Star (DE3) produced no colonies when grown on agar plates, supplemented with IPTG for induction; while if the plasmid was transformed to McrBC-negative *E. coli,* many colonies were observed (460 and 580 in two independent experiments). We suggest that the lack of growth in regular *E. coli* is caused by ParI methylation of the genomic DNA, which is subsequently digested by the McrBC system lead-

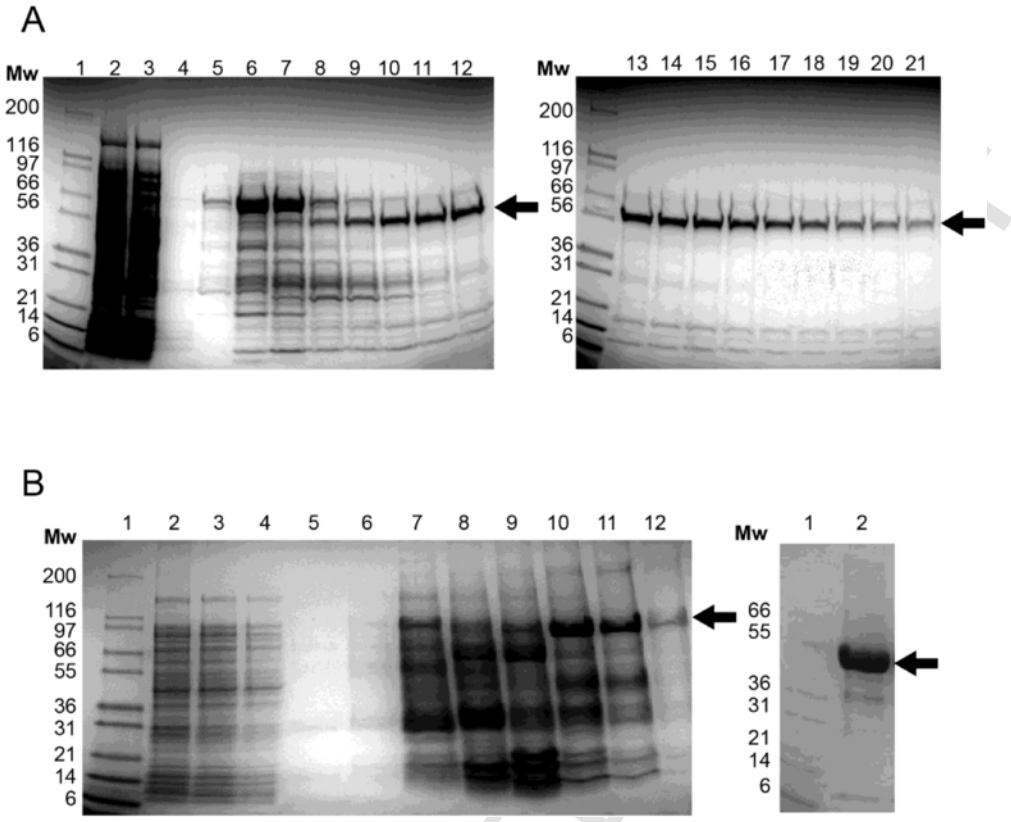

Fig. 3. Purification of recombinant ParI. A. SDS-PAGE analysis of fractions from the initial immobilized metal affinity chromatography (IMAC) purification of the recombinant His-ParI protein. Lane 1, Mark 12 molecular weight marker; lane 2, whole cell extract; lane 3, soluble fraction; lane 4, flow-through fraction from purification; lanes 5–21 fractions from elution across an imidazole gradient. The theoretical molecular weight of His-ParI is calculated to be 54 kDa. **B.** Left: SDS-PAGE analysis of selected fractions from the initial IMAC purification of the recombinant MBP-ParI protein. Lane 1, Mark 12 molecular weight marker; lane 2, whole cell extract; lane 3, soluble fraction; lane 4, flow-through fraction from purification; lanes 5–12, fractions from elution across an imidazole gradient. The theoretical molecular weight of MBP-ParI is calculated to be 94 kDa. Right: SDS-PAGE analysis of fractions from the second IMAC purification step, after removal of His-MBP by TEV protease cleavage. Lane 1, Mark 12 molecular weight marker; lane 2, flow through-fraction containing recombinant ParI after tag-removal. The theoretical molecular weight of ParI is calculated to be 54 kDa.

ing to cell death. This result supports the notion that recombinant ParI is active *in vivo* in *E. coli*. To ensure that the lack of cell growth was not a consequence of low competence or poor viability of the BL21Star (DE3) cell stock, transformations were attempted with plasmids encoding several unrelated proteins, and in all cases these produced colonies.

To investigate the McrBC sensitivity of DNA from the native host of ParI, *P. arcticus* 273-4 genomic DNA was isolated as well as genomic DNA from the related, but C5-DNA-MTase deficient *P.* sp. PRwf-1 strain. After treatment with McrBC restriction enzyme, genomic DNA from *P.* sp. PRwf-1 was found to be intact while genomic DNA from *P. arcticus* 273–4 appeared to be significantly degraded (Fig. 6). This McrBC sensitivity indicated that genomic DNA from *P. arcticus* 273–4 was methylated *in vivo*. According to REBASE, *P. arcticus* contains a second C5-DNA-MTase in addition to ParI, encoded by the gene psyc_0401 (M.ParAORF401P, Table 1). It is possible that this enzyme is responsible for the McrBC-sensitive methylation we observed. However, in light of previous results using recombinant His-ParI (Fig. 6) we can assume that native ParI is able to fully or partially methylate the *P. arcticus* 273-4 genomic DNA, thus, making it sensitive to McrBC. Thus, the results strongly indicate that ParI is a methyltransferase capable of methylating genomic DNA both in its native host and in a heterologous host.

3.6. Recombinant ParI has methyltransferase activity in vitro

In vitro DNA methyltransferase activity was examined by measuring the incorporation of the 3H-methyl group from S-adenosylmethionine (3H-SAM) into DNA using a filter-binding assay at room temperature (Fig. 7). Three versions of the purified protein were tested (MBP-ParI, His-ParI, and ParI after tag-removal) as well as cell lysates of MBP-ParI and His-ParI. For the samples from purified proteins, MBP-ParI is most active, but admittedly this affinity-purified sample has minor impurities and as such there is a small possibility of contaminant activity. Purified His-ParI also showed significant activity. No methyltransferase activity was observed with untagged ParI (the activity of untagged ParI was comparable to the blank in 4 out of 5 purification batches of protein tested with this method). The results indicate that loss of catalytic activity occurred during the purification steps necessary for tag removal. In Fig. 7, the results for the clarified lysates of MBP-ParI (MBP-ParI lysate) and His-ParI (His-ParI lysate) are included as orange bars, but as the specific ParI-concentrations in these samples are unknown, these results should not be over interpreted and strictly speaking can only be used as a qualitative verification of the presence of a DNA-MTase in the samples; however, they accord well with the results for purified proteins.

Prior to this, attempts were made to assess the *in vitro* activity and sequence specificity for untagged ParI methylation using a bisulfite assay; however, the results obtained by this method were inconclusive. Based on the results from the filter binding assay, this was most likely due to the absence of activity for this construct.

4. Discussion

ParI, the first C5-DNA-MTase from a psychrophilic bacterium to be characterized, displays DNA methylation activity *in vivo* and *in vitro*. Its biophysical characteristics are consistent with large regions of disorder

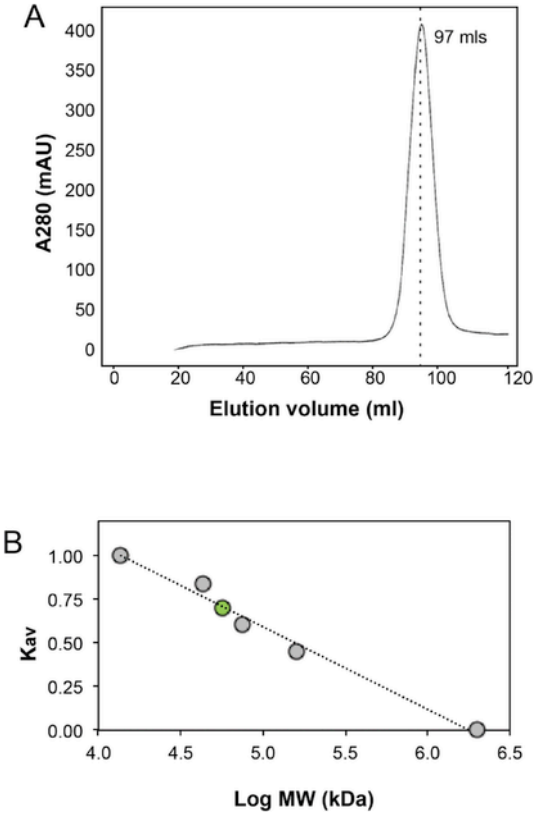

Fig. 4. Gel filtration chromatogram. A. The chromatogram from ParI purification using Superdex 200 size exclusion column shows a single-peak elution at 97 ml. **B.** Calibration curve of the column with log Mw for ParI (green circle). The converted value is 57.5 kDa, which compares well with the calculated molecular weight for ParI of 54 kDa. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

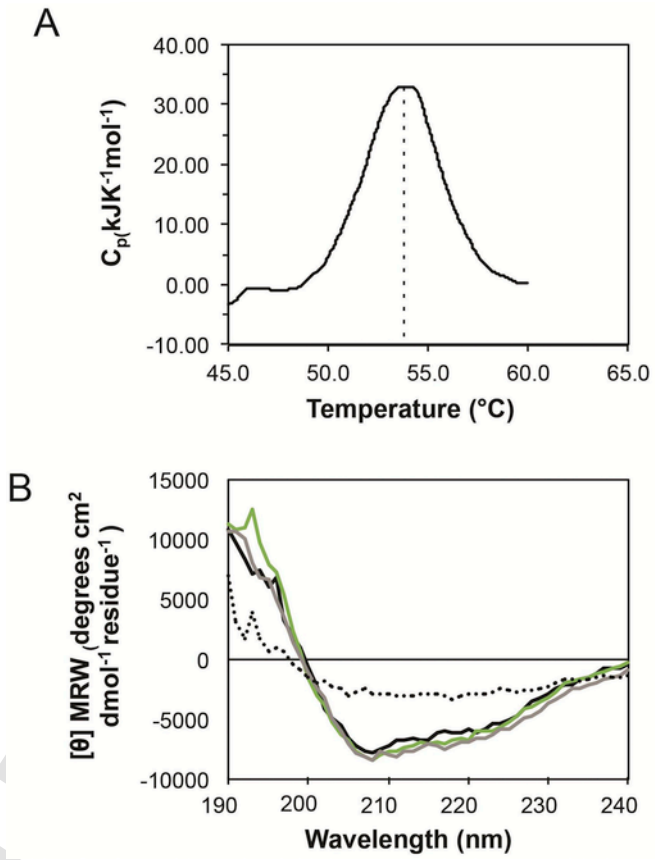

Fig. 5. Thermal stability and unfolding of ParI. A. DSC thermogram for ParI after subtraction of the buffer baseline. The protein was scanned at a rate of 1 °C/min across a temperature range of 5–75 °C; here, only the relevant range for the unfolding event (44–62 °C) is shown. The graph was made in SigmaPlot. **B.** Secondary structure information rendered from CD spectra at 15 °C in the presence of SAM (black line) and in the absence of SAM (green line), as well as at 35 °C (gray line) and at 65 °C (dotted line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including thermal unfolding transitions over broad temperature range and low percentage of secondary structure as measured by CD. ParI unfolds at temperatures typical of many mesophilic proteins, but its temperature optimum could not be determined due to apparent loss of activity after purification. The *in vivo* assays demonstrate toxicity of ParI when recombinantly expressed in *mcr* + *E. coli* at 37 °C, while assays in the native host were carried out at 25 °C, indicating that the enzyme is functional to some extent at both temperatures. Methyltransferase activity was also detected *in vitro* using radiolabelled 3H-SAM and pUC19 as a substrate. Activity was detected both in cell lysates and purified samples, but not after tag removal. The higher activity of MBP-ParI compared to His-ParI, coupled with the complete loss of activity upon MBP removal suggests that the higher specific activity is likely due to increased ParI solubility for this construct. An intriguing point is that, although untagged ParI lost activity after MBP removal and subsequent purification steps, it appears to retain its overall structure. ParI has PCQ-to-DCK substitution in motif IV compared with homologous

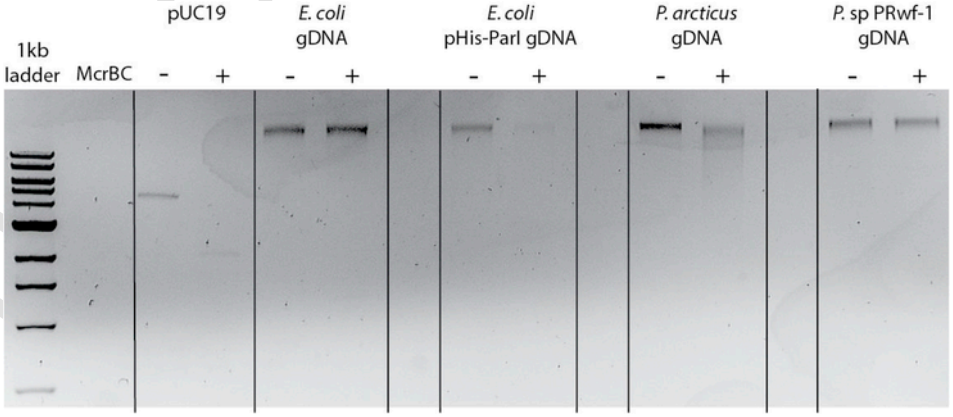

Fig. 6. McrBC sensitivity of ParI methylated DNA. Agarose gel showing McrBC sensitivity of genomic DNA (gDNA) isolated from *E. coli, P. arcticus and P.* sp PRwf-1. A pUC19 plasmid pre-treated with M. SssI methyltransferase was used as positive control for McrBC sensitivity (pUC19). *E. coli* gDNA, empty vector control; *E. coli* pHis-ParI gDNA, *E. coli* gDNA containing pHis-ParI; *P. arcticus* gDNA, gDNA from the parI-containing *P. arcticus*; P. sp PRwf-1, gDNA from the parI-deficient *P.* sp. Prwf-1.

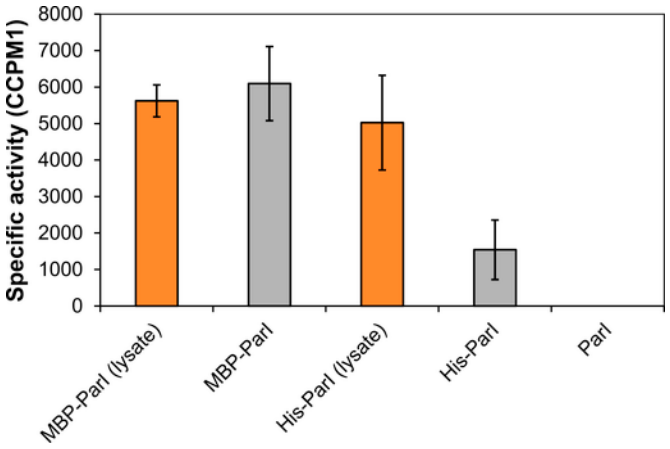

Fig. 7. Methyltransferase activity assay with radiolabelled SAM (3H-SAM). Bar graph showing incorporation of 3H-SAM into DNA by ParI constructs. The measured signal is expressed as blank- and concentration-corrected specific activity in CCPM1 (corrected counts per minute). MBP-ParI (lysate) and His-ParI (lysate) are clarified lysates of soluble protein, while MBP-ParI, His-ParI and ParI are purified proteins. The lysate samples are shown in orange. There is an uncertainty in the ParI-concentrations of the lysate samples, and as such these values can only be used qualitatively.

MTases, where the proline has a role in orientation of the activated cytosine and SAM in order to achieve methyl group transfer in the catalytic reaction [49]. Alterations in this region may make ParI particularly prone to inactivation, while remaining in a somewhat folded state.

Homology searches using BLAST revealed that ParI shares the highest sequence similarity (64%) to an uncharacterized C5-DNA-MTase from *A. baumannii,* which is also of phage origin and shares the motif IV PCQ-to-DCK substitution.

The prevailing scenario for acquisition of orphan MTases is that during evolution, an MTase may have been part of a functional type II RM system that over time lost its REase member due to redundancy [50]. MTases are generally well-conserved during evolution as they methylate at the same or an overlapping site as members of other type II RM system found in bacteria, thereby complementing methylation and protection of host DNA by these enzymes [50]. A similar situation is found in the RM system of *E. coli* where Dcm, an orphan C5-DNA-MTase, has the same recognition sequence as the EcoRII RM system (CCWGG), and it is postulated that Dcm serves as back up for methylation of *E. coli* DNA [50,51]. Our hypothesis however, is that ParI was horizontally acquired as an orphan. Based on our bioinformatics investigations, as well as previously published data [18] we suggest that the gene encoding ParI is of phage origin, most likely a horizontally acquired gene from another bacterium during phage integration, with *A. baumannii* being a possible donor, although the original source still remains elusive. The *par*I gene is situated between two putative phage genes within the *P. arcticus* genome. The gene with the locus tag Psyc_0979, which is upstream to *par*I, is recognized by a BLAST homology search as part of temperate bacteriophage Psymv2 isolated from *Psychrobacter* sp. MV2, whose closest relative is an uncharacterized putative prophage within the *P. arcticus* 273–4 and *A. baumannii* genomes [43]. The region downstream to *par*I, encoding the gene with the locus tag Psyc_0981 is similar to the MU phage [18]. In addition to explaining the orphan status of ParI, horizontal gene transfer of *par*I into *P. arcticus* by a phage may explain why ParI does not exhibit typical cold-adapted features previously documented for other *Psychrobacter* enzymes such as the branched-chain 2-keto acid decarboxylase and murein peptide ligase which have optimal temperature of activity at 30 °C [52,53].

We also note that *P. arcticus* 273–4 is predicted to possesses an incomplete type IV McrBC-like REase, where the C subunit of the canonical BC complex were missing, making the McrB non-functional [54,55]. An evolutionary explanation for this may be that the substrate for a functional McrBC enzyme is methylated cytosine and, assuming that the ParI enzyme is functional, an active McrBC would restrict the methylated genomic DNA, leading to cell death. Therefore the McrBC-negative background of *P. arcticus* made it possible to retain a horizontally transferred methyltransferase. This would be consistent with the results of our activity assays, which showed both that ParI-methylated DNA is sensitive to cleavage by McrBC enzymes, and that transformation with a plasmid encoding ParI was lethal to a McrBC-containing bacterial strain. In addition, the C5-DNA-MTase deficient *P.* sp. PRwf-1 has a putative complete McrBC restriction system encoded in its genome.

5. Conclusions

In this paper we describe the recombinant expression and characterization of ParI, a C5-DNA-MTase from *P. arcticus* 273–4. To our knowledge, this is the first characterization of an orphan C5-DNA-MTase from a psychrophilic bacterium. The C5-DNA-MTase could not be expressed in regular *E. coli* expression strains, likely due to the presence of McrBC enzymes that restricted methylated DNA. Successful expression was obtained using an McrBC-negative *E. coli* expression strain. To improve solubility, an MBP tag was added as a fusion partner between the His-tag and ParI. This contributed to an overall increased yield and solubility. Despite originating from *P. arcticus* 273–4, ParI did not exhibit expected cold-adapted features such as a low melting temperature. We hypothesize that the reason for this is ParI's origin from a phage that is not cold-adapted.

Using two different *in vivo* assays where ParI-methylated genomic DNA was treated with McrBC, we showed that both native and recombinant ParI is able to methylate genomic DNA. Using a radiolabeled methyltransferase assay, we showed that ParI possesses methyltransferase activity also *in vitro* but only as a fusion construct with MBP and/or His-tag, while the untagged protein showed no activity.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

None to be declared.

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Authors' contributions

IL, BA and GEKB conceived the study and participated in its design and coordination, helped in data analysis and reviewed the manuscript, MG performed all experiments, interpreted the data and drafted the

manuscript; AW helped in experimental design, data analysis, performed the CD experiment and reviewed the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pep.2018.05.012.

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