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# Recombinant production of A1S_0222 from *Acinetobacter baumannii* ATCC 17978 and confirmation of its DNA-(adenine N6)-methyltransferase activity

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ABSTRACT

*Acinetobacter baumannii* appears as an often multidrug-resistant nosocomial pathogen in hospitals worldwide. Its remarkable persistence in the hospital environment is probably due to intrinsic and acquired resistance to disinfectants and antibiotics, tolerance to desiccation stress, capability to form biofilms, and is possibly facilitated by surface-associated motility. Our attempts to elucidate surface-associated motility in *A. baumannii* revealed a mutant inactivated in a putative DNA-(adenine N6)-methyltransferase, designated A1S_0222 in strain ATCC 17978. We recombinantly produced A1S_0222 as a glutathione S-transferase (GST) fusion protein and purified it to near homogeneity through a combination of GST affinity chromatography, cation exchange chromatography and PD-10 desalting column. Furthermore we demonstrate A1S_0222-dependent adenine methylation at a GAATTC site. We propose the name AamA (*Acinetobacter* adenine methyltransferase A) in addition to the formal names M.AbaBGORF222P/M.Aba17978ORF8565P. Small angle X-ray scattering (SAXS) revealed that the protein is monomeric and has an extended and likely two-domain shape in solution.

## 1. Introduction

*Acinetobacter baumannii* is a gram-negative opportunistic pathogen that causes nosocomial infections including pneumonia and bloodstream infections which is associated with an increased mortality and multi-drug resistance [1–3]. *A. baumannii* was rated as one of the critical priority 1 pathogens for the development of new antibiotics by the WHO in 2017 [4]. Despite the name “Acinetobacter” meaning non-motile bacteria and despite the lack of flagella, members of the genus are able to move [5,6]. At least two forms of motility are known for *Acinetobacter* species. The so called “twitching motility” depends on retraction of type IV pili [7–9]. Another form of movement, the surface-associated motility, occurs at the surface of semi-dry media and is independent of type IV pili [10]. Almost all tested clinical isolates can move along surfaces [11] and a number of genes required for this form of motility have been identified [10,12,13].

Motility is known to be affected by epigenetic regulation in various bacteria [14]. Epigenetics deals with heritable changes in gene expression without any changes in the DNA sequence. In bacteria the most studied epigenetic mechanism is DNA methylation [15] that is performed by DNA methyltransferases [16]. These enzymes transfer methyl groups from S-adenosyl-L-methionine (SAM) to adenine or cytosine bases. This process protects DNA against digestion from restriction endonucleases and is important for the regulation of various physiological processes such as mismatch repair and transcription [17,18]. Most DNA methyltransferases are part of a restriction-modification system (R-M system). In this case the host DNA gets methylated by the DNA methyltransferase that protects the DNA against digestion by the corresponding endonuclease [19]. In addition, “orphan” methyltransferases are known that act without any associated endonuclease [17]. The most studied orphan DNA adenine methyltransferase called Dam was found in *E. coli* and was shown to methylate adenine bases at GATC sites [20,21]. *Salmonella enterica dam* mutants exhibit a reduced motility [22] and *dam* overexpression in *Yersinia enterocolitica* resulted in an increased motility [23]. The *Acinetobacter baumannii* genome encodes a putative DNA-(adenine N6)-methyltransferase, designated A1S_0222 in strain ATCC 17978 that seems to act without a corresponding endonuclease. We hypothesized that the putative DNA

*Abbreviations:* SAXS, small angle X-ray scattering; DESY, Deutsches Elektronen-Synchroton; GST, glutathione S-transferase; m6A, methylation of the adenosine base at the nitrogen-6 position

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adenine methyltransferase A1S_0222 does impose epigenetic control in *Acinetobacter baumannii* and since little is known about orphan methyltransferases we approached its characterization.

## 2. Materials and methods

**Bacterial transformation and the generation of a *A. baumannii* ATCC 17978 mutant.** Transformation was performed by electroporation [24]. The EZ-Tn5™ < KAN-2 > transposon mutants in *A. baumannii* ATCC 17978 were generated by using the EZ-Tn5™ < KAN-2 > insertion kit (Epicentre Biotechnologies) as previously described [11].

**Surface-associated motility.** Motility plates were composed of 0.5% agarose (w/v), 5 g/L of tryptone, and 2.5 g/L of NaCl as previously described [11]. A single colony from a nutrient agar plate (Oxoid) or selective agar plates (supplemented with 50 μg/mL of kanamycin for the A1S_0222 mutant) of either wild-type or *A. baumannii* ATCC 17978 mutant was taken with the pipette tip and then the surface of a motility plate was touched. Pictures were taken after incubating the plates for 16 h at 37 °C.

**Construction of protein expression plasmids.** The *a1s_0222* gene of *A. baumannii* ATCC 17978 was amplified by PCR using the oligonucleotides *0222-pGEX-6P-3-for*: 5′-ATTAGGATCCAATTCAGAGCCTTC GGTATACCAC-3' (BamHI restriction site underlined) and *0222-pGEX-6P-3-rev*: 5′-ATTAGCGGCCGCTTACCAAAGTGCGAGCTGTGTAC-3' (NotI restriction site underlined). The amplified *a1s_0222* gene was inserted into pGEX-6P-3 expression vector (GE Healthcare) after digestion of insert and vector with BamHI and NotI restriction enzymes. The pGEX-6P-3 expression vector carries a glutathione S-transferase (GST-tag) gene, a PreScission protease restriction site, an ampicillin resistance ($Amp^R$) cassette, encodes a *tac* promotor and is inducible with isopropyl β-D-1-thiogalactopyranoside (IPTG). The plasmid (pGEX-6P-3-A1S_0222) was confirmed by DNA sequencing and transformed into *E. coli* BL21 (DE3) pLysS expression strain.

**Protein expression and purification.** A 3 mL overnight culture of *E. coli* BL21 (DE3) pLysS pGEX-6P-3-A1S_0222 was grown in Luria-Bertani (LB)-Medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.4, 100 μg/mL Amp) at 37 °C and 160 rpm. The overnight culture was diluted 1:100 into 200 mL LB (supplemented with 100 μg/mL Amp) and cultured in a 2 L bottle-flask at 20 °C and 160 rpm for 5 h. Expression was induced by addition of 0.05 mM IPTG and cultures were incubated for 16 h at 20 °C and 160 rpm. Cells were then harvested by centrifugation (10,000 × *g* for 30 min at 4 °C) and the resulting cell pellets were frozen at −80 °C. Cell pellets were solubilized at 4 °C in 20 mL disruption buffer (300 mM NaCl, 1 mM DTT (1,4-dithiothreitol), 5 mM EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetra-acetic acid), 1 mM EDTA (2,2′,2″,2‴-(ethane-1,2-diyldinitrilo) tetra-acetic acid), 2 μL benzonase nuclease (250 U/μL), pH 7.4. The cells were lysed by applying three passages through an EmulsiFlex-C3 homogenizer (Avestin). Lysates were then centrifuged at 20,000 × *g* for 50 min at 4 °C. The soluble lysate was then added to a GSTPrep™ FF 16/10 column (GE Healthcare Life Sciences).

**GST affinity chromatography.** The GSTPrep™ FF 16/10 column (bed volume 20 mL) was equilibrated with 5 column volumes (CV) of GST binding buffer (300 mM NaCl, 1 mM DTT, 5 mM EGTA, 1 mM EDTA, 5% glycerol (v/v), pH 7.4). The soluble lysate was added to the column and washed with 10 CV of GST binding buffer and eluted with 7 CV of GST elution buffer (300 mM NaCl, 50 mM Tris, 1 mM DTT, 5 mM EGTA, 1 mM EDTA, 5% glycerol (v/v), 10 mM reduced L-glutathione (GSH reduced), pH 7.4). Eluted fractions were analyzed by SDS-PAGE and appropriate fractions were pooled. The GST fusion protein was then incubated for 16 h at 8 °C with 40 units PreScission protease per 14.52 mg recombinant A1S_0222 to cleave off the GST tag (26 kDa). After incubation the pooled fractions were centrifuged at 20,000 g for 20 min at 4 °C and diluted 1:4 in dilution buffer (50 mM Tris, 1 mM EDTA, 1 mM DTT, 5% glycerol (v/v)).

**Cation exchange chromatography (CEC).** The HiTrap™ SP XL (GE Healthcare Life Sciences) column (bed volume 1 mL) was equilibrated with 10 column volumes (CV) CEC binding buffer (50 mM NaCl, 50 mM Tris, 1 mM DTT, 1 mM EDTA, 5% glycerol (v/v)). The protein solution was loaded on the column and the column washed with 5 CV CEC binding buffer and subsequently eluted with 30 CV in a linear gradient from 0% to 100% of CEC elution buffer (1000 mM NaCl, 50 mM Tris, 1 mM DTT, 1 mM EDTA, 5% glycerol (v/v)). Eluted fractions were analyzed by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), pooled and loaded on a PD-10 desalting column.

**PD-10 desalting column.** The PD-10 desalting (GE Healthcare Life Sciences) column was equilibrated with buffer B1 (150 mM NaCl, 10 mM Tris, 1 mM DTT and 5% glycerol (v/v), pH 7.4). Further steps were performed as described in the column manual using a gravity desalting protocol. The A1S_0222 protein was eluted with buffer B1. Pooled fractions were concentrated using the Vivaspin® concentrator with a molecular weight cutoff of 10,000 Da and a Hydrosart (HY) membrane (Sartorius Stedim Biotech). The purity of A1S_0222 was analyzed by reducing SDS-PAGE. Protein concentrations were determined using the Bradford method. Purified A1S_0222 was directly used for further analysis or stored at −80 °C.

**Confirmation of the A1S_0222 DNA methylation recognition site.** To confirm the A1S_0222 DNA methylation recognition site, the 800 base-pair (bp) PCR product, Int1, consisting of the 5′-end of the *a1s_0222* gene from *A. baumannii* ATCC 17978, was incubated with and without purified A1S_0222 at 37 °C for 1 h (3 μL Int1 (about 200 ng/μL), 2 μL B1 buffer, 4 μL of 800 μM S-adenosyl-L-methionine (SAM), 3 μL A1S_0222 (2.46 μg/μL), 8 μL RNase free water). Int1 was amplified with the oligonucleotides *Int1-for*: 5′-GGATCCGGATGAAATGATCAGT TATGTGGC-3′ and *Int1-rev*: 5′-CGCTCTAATGCTGTTTGTGTACG-3'. Samples of methylated and non-methylated Int1 DNA were used for Sanger DNA sequencing and analyzed at the Robert Koch-Institute sequencing lab (Berlin, Germany) on an ABI PRISM analyzer. The sequencing chromatograms were analyzed as described previously [25].

**DNA methylation assay.** To test the biological activity of purified A1S_0222, a methylation assay was performed using the Int1 DNA. As an alternative DNA substrate, Seq3 (located in gene A1S_0965 of *A. baumannii* ATCC 17978) was amplified from *Acinetobacter baumannii* 29D2 using the oligonucleotides *Seq3-for*: 5′-GAAGTCACTGATACCAA GGAAGGTATTCATTTTG-3′ and *Seq3-rev*: 5′-GTCTGGAAAATGCTGTG TTTCTAATGCTAG-3' (801 bp). Each fragment contains one *Eco*RI restriction site (G↓AATTC). For the methylation reaction 1 μg of Int1 or Seq3, 2 μL B1 buffer, 4 μL of 800 μM SAM, 8 μL RNase free water and 3 μL A1S_0222 (2.46 μg/μL) or 40 units EcoRI methyltransferase (New England BioLabs) were mixed and incubated for up to 1 h at 37 °C. After incubation A1S_0222 or EcoRI methyltransferase was inactivated by 95 °C for 5 min and Int1 or Seq3 were purified with Hi Yield® Gel/PCR DNA Fragment Extraction Kit (SLG®) according to the manufacturer's instructions and eluted in 10 μL RNase free water. Subsequently, an EcoRI restriction reaction was performed (5 μL purified Int1 or Seq3, 1 μL EcoRI, 2 μL EcoRI buffer, 12 μL RNase free water) for 2 h at 37 °C. For visualization, agarose electrophoresis was carried out at 110 V for 50 min in TAE buffer (agarose concentration: 1.25% (w/v)). GelRed™ (GeneON) was used for gel staining. GelRed™ stock solution was diluted 1:5000 into the agarose gel solution. As a size standard GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) was used. The methyltransferase activity was calculated based on the definition that 1 unit (U) of enzyme converts 1 μmol of substrate per minute at 37 °C.

**Small angle X-ray scattering (SAXS).** Synchrotron SAXS measurements ($I(s)$ vs $s$, where $s = 4\pi\sin\theta/\lambda$; $2\theta$ is the scattering angle and $\lambda = 0.125$ nm) were performed at the EMBL-P12 bioSAXS beam line at the PETRA III storage ring (DESY, Hamburg) as described in Ref. [26] under continuous-flow batch mode operations at 10 °C utilizing an automated robotic sample changer [27]. The scattering intensity data were recorded from 25 μl aliquots of A1S_0222 (2.4 mg/mL) and a