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Expression and purification of the Sgm protein from *E. coli*

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Abstract: The *sgm* gene from *Micromonospora zionensis*, the producer of the aminoglycoside antibiotic G-52, encodes for Sgm methylase which modifies the target site on 16S rRNA and thus protects the producer against its own toxic product. The *sgm* gene was modified by polymerase chain reaction (PCR) and cloned in the QIA*express* pQE-30 vector in order to make a construct that places the $(His)_6$ tag at the N-terminus of the protein. The resulting expression construct was transformed in the *E. coli* strain NM522 and the functional activity of the Sgm-His fusion protein was confirmed *in vivo*. Purification of the $(His)_6$-tagged Sgm protein by Ni-NTA affinity chromatography was performed under native conditions and the protein was detected on a sodium dodecyl sulfate polyacrylamide gel. Sgm methylase was purified to homogeneity > 95 %. Polyclonal antibodies raised to purified $(His)_6$-tagged Sgm protein were used to identify this protein by Western blot analysis.

Keywords: Sgm methylase, *Micromonospora zionensis*, expression, *E. coli*, purification.

INTRODUCTION

Members of the order *Actinomycetales* produce a large number of useful secondary metabolites with pharmaceutical application such as antibiotics, antitumor agents, immunosuppressants, anthelmintics, enzyme inhibitors, and agricultural fungicides, insecticides and herbicides.[1]

The actinomycete *Micromonospora zionensis* produces G-52 (4,6-disubstituted deoxystreptamine aminoglycoside), an unsaturated aminoglycoside antibiotic, which is closely related to sisomicin. This strain, like many other aminoglycoside-producing *Micromonospora* strains, protects itself against its own product by modification of the target site, *i.e.*, ribosomes.[2] An aminoglycoside-resistance determinant from *M. zionensis* was cloned in *S. lividans* and it was shown that the resistance mechanism involves methylation of the 30S ribosomal subunit.[3] The cloned gene was designated *sgm* (sisomicin-gentamicin resistance methylase). It was shown that the *sgm* gene is

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regulated at the translational level by negative autoregulation.[4] Namely, Sgm methylase binds to a specific regulatory sequence in front of the ribosomal binding site on its own mRNA and thus prevents its own further translation when all ribosomes are methylated. The *sgm* gene has also been expressed in *E. coli* under *lacZ* promoter. Due to the translational regulation, the Sgm methylase was not detectable on a sodium dodecyl sulfate polyacrylamide gel, despite the fact that *E. coli* cells containing the plasmid were gentamicin resistant. In order to isolate the Sgm protein, it was decided to clone the *sgm* gene without its regulatory region. Using the QIA*express* system (Qiagen), the Sgm protein was expressed with a $(His)_6$ tag on the N-terminus, which made it suitable for a one step purification by immobilized-metal affinity chromatography.[5]

EXPERIMENTAL

Bacterial strains and culture conditions

Strain *Escherichia coli* NM522 (*supE*, *thi*, Δ(*hsdMS-mcrB*), Δ (*lac-proAB*), F' (*proAB*$^+$, *lacI*^{q}, Δ*lacZ*M15) was used.[6] A Luria-Bertani broth (LB – 10 g tryptone, 5 g yeast extract and 5 g NaCl per 1l, pH 7.4) was used as a rich medium and contained 15 g l^{-1} agar when used as a solid medium.[7] Antibiotics ampicillin and gentamicin were added at standard concentrations to the medium for bacteria harboring recombinant plasmids.

Recombinant DNA techniques

All routine DNA manipulation techniques, including plasmid preparation, restriction enzyme digestions, bacterial transformations, ligations and gel electrophoresis were performed according to Sambrook *et al.*[8] The restriction enzymes were obtained from BRL (Bethesda, Maryland) or Pharmacia (Uppsala, Sweden) and were used according to the manufacturer's instructions.

Modification of the 5' end of the *sgm* gene was done by PCR using two pairs of primers. The first pair consisted of Hist2 (5'-ATGACAAAATGACGGCACCTGCGG-3') and "130" (5'-GCGGCAGGAAGGCGCCG-3'), and the second pair consisted of Hist1 (5'- GCGGATCCGATGACG-3') and "130". This pair of primers introduces the *Bam*HI restriction site (underlined) The PCR reaction was performed in a 50 μl reaction mixture (10 mM Tris-HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 100 pmol of each primer, 2 U of Taq polymerase (Pharmacia) and 50 ng of plasmid DNA) under the following amplification profile: initial denaturation at 96 ºC (10 min) followed by 35 cycles of 94 ºC (1 min), 54 ºC (1 min), 72 ºC (1 min) and a final extension step at 72 ºC for 10 min.

After PCR amplification with first pair of primers followed by the second pair of primers, the 200 bp PCR product was purified from the gel,[8] digested with *Bam*HI and *Bgl*II and ligated with a rest of the *sgm* gene in pUMK-33,[4] creating the pTI-27 plasmid. The pTI-27 was digested with *Bam*HI/*Hind*III restriction enzymes and the 1-kb fragment containing the *sgm* gene was ligated into *Bam*HI/*Hind*III sites of the pQE-30 vector (Qiagen). The resulting construct pQES-5 was transformed into *E. coli* NM522 and the clones were selected by ampicillin and gentamicin resistance.

Protein purification procedure

For overproduction of $(His)_6$-tagged Sgm protein, cells harboring the pQES-5 plasmid were grown in LB medium supplemented with 100 μg/ml ampicillin. 100 ml medium was inoculated with 5 ml fresh overnight culture and incubated at 37 ºC until an optical density of 0.6 at 600 nm was reached. Protein expression was then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the incubation was extended for an additional 4 h. The cells were harvested by centrifugation (5000 × g, 15 min, +4 ºC), resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) with 2 mg/ml lysozyme and lysed by two passages through a French press at 16000 psi. The cell extract was centrifuged (15000 × g, 30 min, 4 ºC) and the supernatant filtered through a 0.22

μm-pore-size filter and applied onto a Ni-nitrilotriacetic acid agarose (Qiagen) column equlibrated with lysis buffer. Chelate affinity chromatography was performed under native conditions according to the standard procedures recommended by the manufacturer (Qiagen).

Purification of the Sgm protein was verified on SDS-PAGE by Coomassie Brilliant Blue R250 staining.

Protein concentrations were determined by the method of Bradford,[9] with bovine serum albumin as the standard.

Western blot analysis

Western blot was performed as described by Burnette.[10] Crude cell extracts of *E. coli* NM522 and *E. coli* transformed with expression plasmid pQES-5 and purified proteins, were loaded on SDS-PAGE (12.5 %), and the separated proteins were then transferred onto a nitrocellulose membrane using a Semi-Dry system Multiphor II (Pharmacia) for 1 h at 14 mA. The membrane was blocked with 5 % non-fat dried milk in washing buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05 % Tween 20) and was then subjected to immunoreaction with 500-fold-diluted rabbit immune serum containing polyclonal anti-Sgm antibodies. The secondary antibody (goat anti-rabbit immunoglobulin G) conjugated with alkaline phosphatase was used at a 1: 8000 dilution (Sigma). Immunoblots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) as a color substrate according to the manufacturer's instructions (Promega).

RESULTS AND DISCUSSION

Subcloning of the sgm *gene into a pQE-30 vector*

The *sgm* gene is autogenously regulated at the translational level and thus the Sgm protein is present within the cell at very low concentrations, sufficient to provide resistance. The strategy to isolate Sgm protein was to construct a plasmid which places the $(His)_6$ tag at the N-terminus of the protein. To begin with construction of a plasmid en-

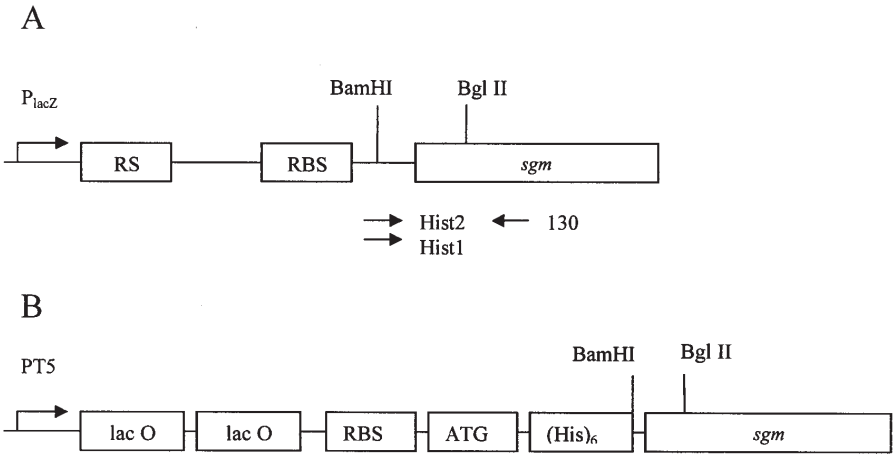

Fig. 1. Scheme of a wild type *sgm* gene and its regulatory region with designated positions of the PCR primers used for amplification of the desired DNA fragments, and *Bam*HI and *Bgl*II restriction sites used for cloning (A). Scheme of the pQES-5 expression construct representing differences in the 5' region of the cloned *sgm* gene (B). For details see Experimental section. Abbreviations: P_{lacZ} – promoter of *lacZ* gene, RS – regulatory sequence of *sgm* gene, RBS – ribosome binding site, PT5 – phage T5 promoter, *lac* O – lactose operator sequences, ATG – start codon, $(His)_6$ – $(His)_6$ tag sequence.

coding a *his*-tagged *sgm* gene, the 5' end of this gene was modified by the PCR technique. Forward primers were designed to introduce a *Bam*HI restriction site and codons for 5 additional amino acids at the N-terminus which are the recognition site for enterokinase.[11] Using enterokinase, the $(His)_6$ tag can be easily removed. Modified 5' end of *sgm* gene digested with *Bam*HI and *Bgl*II was ligated with the rest of the *sgm* gene in pUC19, creating a pTI-27 plasmid. A 1-kb *Bam*HI/*Hind*III fragment containing *sgm* from the resulting constructs (pTI-27) was cloned into the *Bam*HI/*Hind*III sites of the pQE-30 vector which places the $(His)_6$ tag at the N-terminus of the protein (Fig. 1A and 1B). The final plasmid pQES-5 was transformed into *E. coli* strain NM522 cells for expression of the *sgm* gene. Gentamicin resistance of *E. coli* cells containing recombinant plasmid pQES-5 confirmed the functionality of the Sgm-His fusion protein *in vivo*, *i.e.*, this protein was able to methylate 16S rRNA.

Expression and purification of the Sgm protein

The *Micromonospora zionensis sgm* gene was overexpressed in the *E. coli* strain NM522 under the control of a promoter-operator element consisting of a phage T5 promoter (recognized by the *E. coli* RNA polymerase) and the two *lac* operator sequences which bind the *lac* repressor and ensure an efficient repression of the powerful T5 promoter in *E. coli*,[12] resulting in an observable overproduction of the protein in crude extracts of the recombinant strain. Expression of the *sgm* gene gives rise to a major band on an SDS-polyacrylamide gel, corresponding to a 32 kDa protein. This mo-

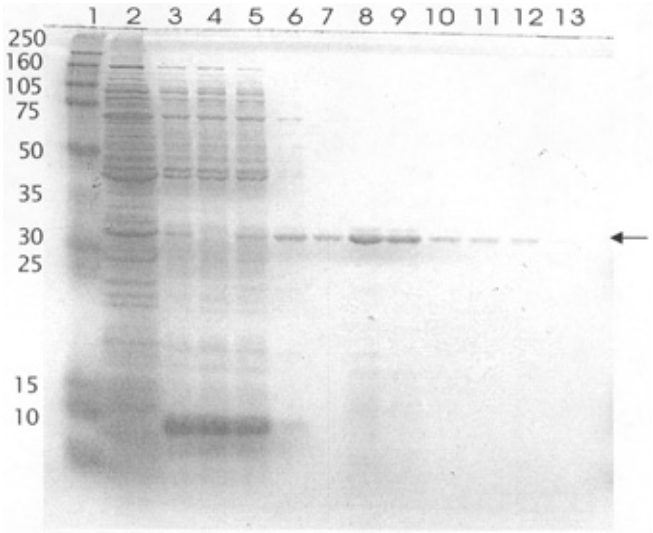

Fig. 2. Purification of recombinant $(His)_6$-Sgm protein under native conditions using Ni-NTA affinity chromatography (for details see Experimental section). A Coomassie-stained SDS-12.5 % polyacrylamide gel is shown. Lanes: 1-protein molecular size standards (in kDa), 2- crude extract of *E. coli* pQES-5 (non-induced control), 3- cell lysate of *E. coli* pQES-5 2 h after addition of IPTG, 4- flow-through, 5-, 6-, and 7- wash with 20 mM imidazole-containing buffer, 8-, 9-, 10-, 11-, 12-, and 13- elution with 250-mM imidazole-containing buffer. The arrow indicates recombinant Sgm.

lecular mass is consistent with the calculated size (31 kD) for the predicted Sgm protein[13] plus the extra residues corresponding to the tail used for its purification. The recombinant Sgm was highly purified by means of a Ni-NTA agarose column, which was verified by SDS-PAGE (Fig. 2). The purified Sgm protein was then used for the production of a rabbit polyclonal antiserum.

Western blot analysis of a cell extract from E. coli *pQES-5*

In a Western blot, crude cell lysates of *E. coli* pQES-5 were transferred from a polyacrylamide gel to a nitrocellulose membrane and the Sgm protein was detected by using polyclonal anti-Sgm antibodies from rabbit. In this experiment, the positive control was purified Sgm protein and the negative control was *E. coli* strain NM522 (Fig. 3).

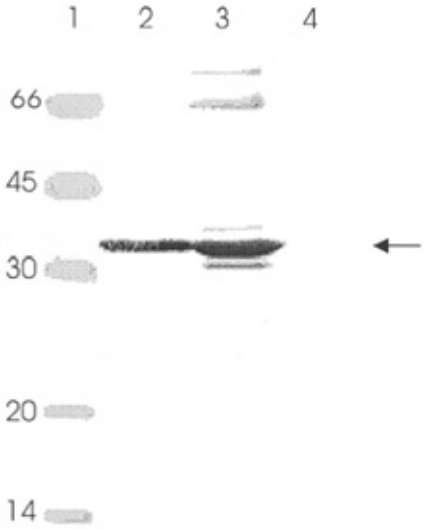

Fig. 3. Western blotting of recombinant Sgm protein using polyclonal anti-Sgm antibodies from rabbit. Lanes: 1– protein molecular size standards (in kDa), 2– purified Sgm protein, 3– *E. coli* pQES-5 crude cell extract, 4– *E. coli* strain NM522 crude cell extract. The arrow indicates Sgm protein.

As the Sgm protein was detected in *E. coli* transformed with the pQES-5 plasmid, polyclonal anti-Sgm antibodies were used to detect recombinant Sgm protein in *Saccharomyces cerevisiae*, *i.e.*, to analyse expression of the *sgm* gene in a eukaryotic system. Purification of Sgm protein was important for further studies, such as *in vitro* analysis of translational autoregulation (RNA gel shift experiments) and for *in vitro* methylation of 40S ribosomal subunits. Although the functionality of the $(His)_6$-Sgm protein was proven *in vivo*, for all other experiments, it is possible, if necessary, to remove the $(His)_6$ tag by digestion with enterokinase.

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ИЗВОД

ЕКСПРЕСИЈА И ПРЕЧИШЋАВАЊЕ Sgm ПРОТЕИНА ИЗ *E. coli*

ТАТЈАНА ИЛИЋ ТОМИЋ, САНДРА МАРКОВИЋ и БРАНКА ВАСИЉЕВИЋ

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Из соја *Micromonospora zionensis*, произвођача антибиотика G-52, клониран је *sgm* ген који кодира специфичну 16S rRNK метилазу одговорну за метиловање циљног места, па самим тим и за заштиту произвођача од сопственог токсичног продукта. Ген *sgm* је најпре реакцијом ланчане полимеризације модификован на 5´ крају како би се омогућило клонирање у QIAexpress pQE-30 експресиони вектор. На овај начин је N-терминус Sgm протеина обележен са 6 хистидина. Функционална активност $(His)_6$-Sgm фузионог протеина је потврђена *in vivo*. Пречишћавање His-обележеног протеина метал-афинитетном хроматографијом је урађено под нативним условима и протеин је детектован на SDS полиакриламидном гелу. Sgm метилаза је пречишћена до хомогености > 95 %. Поликлонска антитела добијена на $(His)_6$-обележен Sgm протеин су коришћена у Western blot анализи.

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