SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Analysis of physiological role of protein mono-ADP-ribosylation in *Streptomyces coelicolor*

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2012

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The Examination takes place at the Library of the Department of Physiology, Medical and Health Science Center, University of Debrecen
30th April, 2013

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
30th April, 2013

1. INTRODUCTION

1.1. The *Streptomyces coelicolor* A3(2)

Streptomyces species are soil-dwelling Gram-positive bacteria that belong to the order *Actynomycetales*. They are well known for their complex life cycle. On solid medium spores germinate to produce a complex substrate mycelium of apically growing filamentous multigenomic hyphae. At a given stage of the life cycle substrate mycelium gives rise to aerial hyphae, which subsequently undergo septation to form chains of monogenomic, thick walled reproductive spores. The *bld* cascade (for bald, meaning unable to form aerial hyphae) regulates initiation of aerial growth, resulting in the formation of surface active molecules (SapB peptide, chaplins, rodlins) that lower the water surface tension and enable hyphae to grow into the air, whereas the *whi* genes control spore formation. Morphological differentiation process is coupled to the production of a wide variety of secondary metabolites (such as antibiotics) with notable medical, pharmacological, biotechnological importance. *Streptomyces coelicolor* A3(2) (*S. coelicolor*) is one of the most well studied model streptomycete. *S. coelicolor* produces several antibiotics, among them the blue-pigmented actinorhodin that is secreted by the *act* transport system. Complete understanding of the regulation of differentiation process and antibiotic production would require the characterization of regulatory mechanisms acting on protein level.

1.2. Mono-ADP-ribosylation and mono-ADP-ribosyltranferase (ADPRTase) enzymes

Mono-ADP-ribosylation of cellular proteins can occur through enzymatic and non-enzymatic mechanisms. The enzymatic reaction is catalysed by **mono-ADP-ribosyltranferase** (**ADPRTase**) enzymes. Mono-ADP-ribosylation of proteins is a phylogenetically ancient post-translational modification in which a single ADP-ribose moiety of β-NAD^+ is transferred to a specific amino acid side chain of a target protein. It is a reversible regulatory mechanism because the inhibitory effect of this modification on protein function could be reversed by removal of the ADP-ribose moiety by ADP-ribose-protein glycohydrolases.

The best-known mono-ADPRTases are bacterial toxins, such as cholera, diphtheria, or pertussis toxin which exclusively act on eukaryotic target proteins, resulting in permanent

activation or inactivation of the cell functions modulated by these crucial host cell protein substrates. Ectogenous and endogenous ADPRTases have also been detected in various prokaryotes, archeas, and eukaryotes, too. ADP-ribosylation of DNA on the amino group at *N*2 of 2'-deoxyguanosine by pierisins, apoptosis inducing cytotoxic proteins of *Pieris rapae* and *P. brassicae* was also described. Pierisins show homology to the ADP-ribosylating mosquitocidal toxin produced by *Bacillus sphaericus*.

Although similarities between the primary sequences of ADPRTases are usually very limited their structural conservation is high. Prokaryotic ADPRTases fall into two broad groups – the diphtheria toxin (DT) group and the cholera toxin (CT) group – based on the characteristics of the conserved consensus sequences. All known ADPRTases share three conserved regions that form the NAD^+-binding and catalytic site: (i) N-terminal region 1, which contains a conserved Arg (in the DT group His replaces Arg) in a β sheet and which is involved in the formation of the NAD^+ binding site and active-site integrity; (ii) a central region 2, containing the aromatic-hydrophobic-Ser-Thr-Ser motif (replaced by two Tyr residues in the DT group) that is characterized by a β sheet followed by an α-helix important for positioning the NAD^+ substrate; and (iii) a highly acidic region 3, the ADP-ribosylating turn-turn (ARTT) loop that contains the strictly conserved catalytic glutamate, which is crucial for catalysis and associated with a nonpolar β strand responsible for the transferase activity. Common substrate recognition domains have not yet been identified.

Endogenous bacterial mono-ADP-ribosylation reactions are also occur to control metabolic enzymes. This is the case in the photosynthetic nitrogen-fixing bacterium *Rhodospirillum rubrum*, where mono-ADP-ribosylation regulates the nitrogenase enzymes responsible for nitrogen fixation, this was the first clear demonstration of physiological role of this reaction. Mono-ADP-ribosylation is catalysed by a dinitrogenase reductase *arginine*-specific mono-ADP-ribosyltransferase (DRAT). The dinitrogenase reductase is fully reactivated by a specific ADP-ribosylarginine hydrolase called dinitrogenase reductase-activating glycohydrolase (DRAG) that hydrolyses the *N*-glycosidic bond of the ADP-ribosylated protein. The activities of DRAT and DRAG are known to be controlled *in vivo*.

Although many ADP-ribosylated proteins have been identified from different bacterial species, little is known of the endogenous ADPRTases and their physiological role in bacterial cells.

1.3. Mono-ADP-ribosylation in *Streptomyces* species

Previously, our research group demonstrated for the first time the presence of ADP-ribosylated proteins in Gram positive bacteria, first in *S. griseus* later in *S. coelicolor*. We have shown Arg-specific ADPRTase activity in *S. griseus* using agmatin as ADP-ribose acceptor but we had no knowledge about the ADPRTase enzymes themselves and the ADP-ribosylated proteins for a long time. The first identified ADP-ribosylated protein in *S. griseus* was glutamin synthetase (GS). The subunits of the enzyme could be ADP-ribosylated and adenylylated too. Both modification resulted in inactivation of GS activity.

Using inhibitors of ADPRTase enzymes (m-aminophenylboronic acid, 3-aminobenzamide) our experimental results suggested that ADP-ribosylation may have significant role in the development and secondary metabolite production in *Streptomyces*. We have found that the *brgA* mutant *S. coelicolor* strain showed defects in protein ADP-ribosylation, aerial mycelium formation and actinorhodin production. The *brgA* mutation as a new pleiotropic regulatory locus was mapped near *uraA* locus.

A japanese group identified four proteins ADP-ribosylated on a cysteine residue (MalE, MldBK, a periplasmic protein for binding branched-chain amino acids, and a periplasmic solute binding protein) from *S. coelicolor*. All of these proteins participated in the transport of metabolites or nutrients through the membrane, and contain liporotein modification site with cysteine residue which is the suggested site of the ADP-ribosylation.

Knowing the complete genome sequence the *SCO2860 (arr)* gene was identified as an ADPRTase coding gene. The gene product Arr-sc is homologue of the rifampin ADP-ribosyltransferase, Arr-ms in *Mycobacterium smegmatis*. Arr-ms not only inactivate the antibiotic but also has role in mycobacterial response to DNA double strand breaks, therefore Arr-sc may have unexplored biological function in *S. coelicolor.*

2. AIMS

Althought our previous results suggest that the ADP-ribosylation may play significant role in growth and differentiation of *Streptomyces* species, our knowledge was limited about the ADP-ribosylated proteins and the enzymes, which catalyze the modification. In our study we have focused on the physiological role of endogenous mono-ADP-ribosylation in the well-known model streptomycete organism, the *Streptomyces coelicolor* A3(2) strain.

In order to identify ADP-ribosylated proteins and ADPRTase enzymes and to understand their role in the cell the following experiments were carried out:

1. optimization of the *in vitro* ADP-ribosylation reaction conditions for modification of proteins in crude extract of the *S. coelicolor* M145 wild type strain culture.
2. isolation and identification of ADP-ribosylated proteins from wild type strain.
3. identification of genes coding for putative mono-ADP-ribosyltransferase enzymes in the genome sequence using search for consensus motifs common to ADPRTases furthermore an *in silico* analysis the structure of protein coded by the identified genes.
4. elimination of an ADPRTase coding gene from the genome thus creating a null mutant.
5. analysis of the phenotype of the null mutant
6. complementation of the null mutant with the wild type gene in order to prove that the the mutant phenotype was caused by the absence of the enzyme.
7. comparison of ADP-ribosylated protein pattern of the wild type strain with that of the mutant to demonstrate that the identified gene really codes an ADPRTase enzyme.

3. MATERIALS AND METHODES

3.1. Materials

We used the highest purity chemicals that are commercially available. *E. coli* HB101 was used as the host for cosmid cloning. John Innes Center has provided us with the following bacterial strains and vectors: *S. coelicolor* A3(2) M145, a plasmid-free, wild type, prototrophic, antibiotic producing strain; *E. coli* BW21153/pIJ790 (was used as a host for λ RED recombination-based PCR-targeted gene disruption); *E. coli* ET12567/pUZ8002 (was used as the donor in conjugational DNA transfer into *S. coelicolor* M145); pWHM3 (Amp^R, $Thio^R$) *E. coli*/*Streptomyces* shuttle vector (was used for complementation), and St3D11 cosmid (contains *SCO5461* gene and was used in ReDirect method).

3.2. Methodes

Isolation and identification of ADP-ribosylated proteins from *S. coelicolor*

For setting optimal conditions of ADP-ribosyltransferase assay (adenine-2,8-^{3}H)NAD^+ was used as substrate to measure ADPRTase activity. In brief, 36h old cells were scraped off from the cellophane-covered SFM-agar plates. Cells were disrupted by sonication. Cell debris was removed by centrifugation and in the ADPRTase assay the supernatant was used as the crude cellular extract, which provided not only the enzyme protein but also substrate proteins to be ADP-ribosylated (endogenous ADP-ribosylation). The supernatant was halved and one aliquot was supplemented with ADP-ribose, the other with an equal volume of dH_2O. The ADP-ribosilation reaction was started with the addition of (adenine-2,8-^{3}H)NAD^+ (100 μM) and the samples were incubated at 30^{o}C. At given times small aliquots were removed from both reaction mixtures and their protein content was precipitated with trichloroacetic acid (TCA) at 0^{o}C for 30 min. The radioactivity remaining on the filters was determined with a liquid scintillation counter. For purification of ADP-ribosylated proteins cellular crude extract was prepared as described above. We complemented the solution with ADP-ribose based on the results from previous experiments. Half aliquot was supplemented with NAD^+, the other half with equal volume of dH_2O. After the ADP-ribosylation reaction the precipitated proteins were redissolved and was purified an m-aminophenylboronic acid–agarose affinity

chromatography column. Concentrated proteins from both samples were separated by 2D-gel electrophoresis to obtain a representative pattern of ADP-ribosylated proteins. Coomassie-Brillant Blue stained protein spots were excised from 2D-gels. After in-gel trypsin digestion samples were analyzed by MALDI-TOF mass spectrometry. MALDI-TOF peptide mass fingerprint data were analyzed using the "MASCOT" search engine.

Disruption of *SCO5461* in *S. coelicolor* M145 and complementation of the null mutant

We identified the *SCO5461* as a gene of a putative ADPRTase enzyme in the genome of *S. coelicolor* using bioinformatics approache (PBLAST, PSI-BLAST, StrepDB database). PCR targeted mutagenesis (ReDirect technology) was used to replace the chromosomal *SCO5461* allele with an apramycin resistance marker, *apr* (*aac(3)IV*) generated by PCR using primers with 36 nt homology extension.

First we amplified the required *apr-oriT* cassette from plasmid pIJ773. The PCR product was introduced into *E. coli* BW21153/pIJ790 harboring the cosmid St3D11 and expressing the λ RED recombinase system. The resulting recombinant cosmid, carrying *SCO5461::apr* instead of *SCO5461*, was introduced into *S. coelicolor* M145 by conjugation using *E. coli* ET12567/pUZ8002. The apramycin-resistant, kanamycin-sensitive exconjugants were selected, and gene disruption was confirmed by PCR coupled to restriction fragment length polymorphism analysis (RFLP) of the PCR product using XhoI enzyme.

Complementation of the *S. coelicolor* M145 *ΔSCO5461::apr* mutant was achieved using the plasmid pWHM3. The entire open reading frame of *SCO5461* gene was amplified by PCR with *S. coelicolor* M145 genomic DNA as template. The 636 bp PCR product was inserted between the *Hind*III and *Eco*RI sites of pWHM3 in frame with the weak *lacZ* promoter to obtain pWHE1. The resulting pWHE1 plasmid was cloned in *E. coli* HB101 cells and the construct was verified by PCR and RFLP analysis. pWHE1 was then transformed into *S. coelicolor* M145 *ΔSCO5461::apr* to obtain the thiostrepton resistant complemented *S. coelicolor* M145 *ΔSCO5461::apr*/pWHE1 strain.

Phenotypic analysis of the null mutant

Morphological analysis: In order to compare the morphological differentiation of the mutant strain, the complemented mutant strain and the wild type M145 strain, all of them were cultivated on four different solid media – SFM, SMMS supplemented with 10% sucrose (SMMS+), and R5. For microscopic analysis the cells were grown on coverslips inserted into three different agar plates (SMMS, SMMS+, R5). 2 and 5 days old samples were fixed with methanol. Tracing the development of the strains under a phase-contrast light microscope we confirmed characteristics observed on the plates. The DNA of 2 and 7 days old samples from plates with coverslips were stained with propidium-iodide and studied under confocal laser scanning microscope.

Production and secretion of antibiotic: The strains were grown on sterile filters deposited on the surface of three different solid media (SMMS, SMMS+, R5). The filters did not allow the growth of the mycelium into the medium. Removal of the filter with the mycelium on it after seven days of cultivation makes it possible to visualize the blue actinorhodin antibiotic secreted into the extracellular environment.To analyse extracellular γ-actinorhodin, the wild type and the null mutant strains were cultivated for four days in SFM liquid medium. Cultures supernatants were acidified to pH 2-3 and methanol:chloroform (1:1) was used to extract the antibiotic. The A_{542} was measured in the chloroform phase. The amount of produced antibiotic was normalized to protein content in the sediment.

Protein ADP-ribosylation assay

Comparison of ADP-ribosylated protein pattern in different strains: The mutant, complemented mutant and the wild type strains were grown 32 hours on cellophane-covered R5 plates. In the ADP-ribosylation reaction ADP-ribose and β-[adenylate-^{32}P]NAD^{+} were added to the crude extracts of mycelia. After precipitation with TCA proteins were solubilised and separated on SDS-PA-gel. Finally, proteins from the gel were electrophoretically blotted to Immobilon P transfer membrane in a semi-dry transfer assembly. The labeled proteins on the blot were detected by a phosphor image analyzer.

Sequence identification and alignments; secondary structure prediction and 3D structure prediction

The putative amino acid sequence of the SCO5461 was obtained from the genome database of *S. coelicolor* (http://streptomyces.org.uk/). Sequence analysis and alignments were performed using PSI-BLAST search through the NCBI web server (www.ncbi.nlm.nih.gov/BLAST). Secondary structure predictions were made using SSPRED software (www.coot.embl.de/~fmilpetz/SSPRED) and TMpred server (http://www.ch.embnet.org). Signal peptide analysis was done using SignalP program (www.cbs.dtu.dk/services/SignalP); TaTFind and TatP software (www.signalfind.org and www.cbs.dtu.dk/services/TatP, respectively). Protein 3D structure was predicted by using the $(PS)^2$ modeling software (http://ps2.life.nctu.edu.tw).

Databases

The following databases were used for genomic sequence analysis of the various *Streptomyces* strains: http://www.broadinstitute.org (for *S. lividans; S. albus; S. pristinaespiralis*); http://streptomyces.org.uk (for *S. coelicolor; S. scabies; S. griseus; S. avermitilis; S. clavuligerus, S. venezuelae*).

4. RESULTS AND DISCUSSION

Our group has already demonstrated the presence of ADP-ribosylated proteins and an arginine-specific ADPRTase activity in *S. griseus*, but no exact molecular information was available about ADP-ribosylated proteins or enzymes catalysing this type of modification in *Streptomyces*.

To understand the biological function of protein mono-ADP-ribosylation, *S. coelicolor* we have tried to identify endogenously ADP-ribosylated proteins; to find unknown genes encoding ADPRTase enzymes by sequence homology; (3) to disrupt the gene coding for identified ADPRTase enzyme in order to recognise phenotipical differences between the wild type and the mutant strains.

4.1. Identification of ADP-ribosylated proteins in *Streptomyces coelicolor*

First the optimal conditions of ADP-ribosylating reaction were established. We have showed that the maximal incorporation of ADP-ribose was reached 30 min after the addition of radioactive [^{32}P]-NAD^{+} as a substrate to the cell-extract of *S. coelicolor* then it decreased. The cause of this decrease is probably the NADGH activity of the cell-extract hydrolyses the substrate and the MARH activity cleaving the incorporated ADP-ribose from the proteins making this posttranslational modification reversible. [In the genome of *S. coelicolor* at least six genes showing significant homology with the known MARH enzymes can be found (*SCO0086, SCO1766, SCO2028, SCO2030, SCO4435, SCO5809;* personal sequence analysis).] The incorporation of ADP-ribose into proteins could be increased with the addition of an excess of unlabelled ADP-ribose, which could inhibit the MARH activity (NADGH is not inhibited by ADP-ribose). The great excess of “cold” ADP-ribose also reduces the non-enzymatic incorporation of [^{32}P]-ADP-ribose produced by the NADGH present in the cell extract of *S. coelicolor*.

The *in vitro* ADP-ribosylated proteins of *S. coelicolor* were purified by m-aminophenyl-boronic acid affinity chromatography and separated with 2-D SDS-PA gel electrophoresis. The separated proteins were in gel digested with trypsin, and the peptide patterns were analysed by MALDI-TOF. Eight *S. coelicolor* ADP-ribosylated proteins were identified. Some of the proteins were found in more than one spots with different pI on the 2-D gel. In case of SCO5477, a putative oligopeptide-binding lipoprotein (an ABC transporter subunit)

several isoforms were found with slightly different pI values, probably due to ADP-ribosylation or by spontaneous desamination of Asp or Glu. The other proteins (inosine-5'-monophosphate dehydrogenase, IMPDH, coding by *SCO4771*; and FolD, methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase, coding by *SCO4824*) were found in two spots on the 2-D gel. In both cases one of the spots has the expected molecular weight and pI, the other spot represented a protein variant with the same molecular weight but lower pI. Both IMPDH and FolD function as multimers in the cell, and interestingly, both the unmodified and modified forms of the proteins were found on the 2D gel. This raises the possibility that the activity of the multimer is influenced by the ratio of the ADP-ribosylated and unmodified subunits. The modification-dependence allows a kind of fine-tuning of the enzyme activity of the complex to adapt to current needs of cell metabolism. Similar regulatory mechanism was observed in *E. coli*, in case of glutamine synthetase having twelve identical subunits. The activity of this enzyme complex is regulated by the ratio of unmodified and adenylylated subunits.

According to their suggested or known biological roles the eight proteins can be categorised as (1) secreted proteins: the putative periplasmic branched-chain amino acid-binding protein SCO2008 and SCO5477, and the other two secreted proteins SCO6108 (FusH protein) and SCO1968 (2) metabolic enzymes using NAD^+/NADH or $NADP^+$/NADPH as coenzymes: IMPDH (SCO4771) and FolD (SCO4824), and (3) other proteins: SpaA (coding by *SCO7629*) and glutamine synthetase (GlnA, coding by *SCO2198).*

(1) SCO2008 and SCO5477 are the extracellular ligand-binding lipoprotein subunit of different ABC transporters. Confirming our results SCO2008 was previously identified as a protein ADP-rybosylated on a cysteine side-chain by Sugawara and his colleagues. This ADP-ribosylated cysteine is the one, which is present in the lipobox part of the signal sequence necessary for the translocation and for the lipid modification of the prospective lipoprotein. Sugawara and his colleagues assumed that ADP-ribosylation of the Cys side chain inhibited the secretion of the unmatured protein, so the lipoprotein diacylglycerol transferase could not attaches a lipid to it. ADP-ribosylation would prevent both the export of the protein and its membrane attachment, so the ADP-ribosylated protein remaining in the cytoplasm would not be able to carry out its function in transport processes. Similarly to SCO2008 the SCO5477 protein has a signal sequence containing a cysteine in an appropriate position, thus Piette and his colleagues assumed that SCO5477 is an ADP-ribosylated protein, too. Our findings support this idea.

Both of the other two secreted proteins, SCO6108 (FusH) and SCO1968 are hydrolases. FusH is an esterase providing fusidic acid resistance. Since the ADP-ribosylated FusH was isolated from the cytoplasm, ADP-ribosylation of the protein could influence its export. SCO1968 is a hypothetic secreted hydrolase showing homology with GlpQ, a glycerophosphoryl diester phosphodiesterase in *Bacillus subtilis* induced by phosphate-starvation. The reversible ADP-ribosylation of SCO1968 can be a posttranslational regulatory mechanisms influencing glycerine metabolism in *S. coelicolor*.

(2) Both SCO4771 and SCO4824 are dehydrogenases. In their cases the ADP-ribosylated peptide fragments were identified by MASCOT program. SCO4771 is an inosine-5'-monophosphate-dehydrogenase (IMPDH) in *S. coelicolor*. The intracellular GTP concentration has a crucial role in the regulation of morphological differentiation in prokaryotes. Since IMPDH catalyses the key step in *de novo* GTP biosynthesis, ADP-ribosylation of this enzyme can be an important physiological regulatory mechanism of spore formation and antibiotic production. Analysis of the amino acid sequence data of two tryptic peptides (207-225 and 226-245) from the major spot suggested that IMPDH was modified at two different positions, on Arg-225 and Arg-244 in a highly conserved region. The other dehydrogenase FolD encoded by *SCO4824* is involved in tetrahydropholate metabolism; it has both methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase activity. The GAEVVVVGR peptide fragment of the enzyme, which was found to be ADP-ribosylated in our experiment, is part of the evolutionary highly conserved $NADP^+$-binding motif. ADP-ribosylation and thus inactivation of FolD can have a crucial effect in the regulation of the primary metabolic processes and differentiation through the modification of methionine and purin biosynthesis.

Two proteins belong to the third group. One of them is the *SCO7629* encodes the SpaA protein. This protein is homologous with the RspA in *E. coli* which is involved in the regulation of the stationary growth phase. It is known that the disruption of *SCO7629* gene in *S. coelicolor* causes a conditional, cell density-dependent phenotype. So the endogenous ADP-ribosylation of SpaA can have an effect on the response of the bacterium given to the cell density. SpaA could serve as a starvation state sensor protein. The other gene *SCO2198* codes glutamine synthetase GlnA. Activity of GlnA is regulated by adenylylation in prokaryotes (especially in intestinal bacteria). Hesketh and his colleagues proved the adenylylation of the conserved Tyr-397 side chain of this protein in *S. coelicolor*. Our group showed previously that the *in vitro* ADP-ribosylation of GlnA, which inactivated this glutamine synthetase in *S. griseus*. This modification was also found in *Rhodospirillum*

rubrum and later in *Synecocystis* species. Our result confirmed the presence of ADP-ribosylation of GlnA in *Streptomyces* species.

Six of the eight identified proteins are newly identified targets of ADPRTases. The remaining two findings were validation of previous results. The finding that four proteins (SCO5477, IMPDH, SpaA, and FolD) are ADP-ribosylated could provide evidence for our previous hypothesis about the connection between protein ADP-ribosylation and morphological differentiation.

4.2. Identification of *SCO5461*, presumably encoding an ADPRTase enzyme and bioinformatical analysis of its hypothetical protein product

Since one of our aims was to create an ADPRTase mutant strain we had to identify genes encoding ADPRTase enzymes. We have applied a bioinformatic approach to find possible ADPRTase coding genes in the genome of *S. coelicolor* A3(2). The high sequence plasticity characteristic for ADPRTases makes the *in silico* identification of homologues difficult, so the position-sensitive PSI-BLAST program was used for sequence analysis. The comparison of the full amino acid sequence of the known ADPRTase proteins shows only little identity, but the NAD^+-binding site and the three strongly conserved sequence motifs in the catalytic active center can help in the identification of ADPRTase enzymes. As a result we found two possible ADPRTases encoding genes in the genome of *S. coelicolor*, the above mentioned *SCO2860* and *SCO5461* genes. Based on the comparison narrowed down to the three motifs, the nearest homologues of SCO5461 besides the ADPRTases in *Streptomyces* strains were the DNA ADP-ribosylating pierisin-1 and -2 (found in butterflies, cytotoxic for mammalian cells), the Arg specific MTX in *Bacillus sphaericus* and the catalytic subunit CT-A of cholera toxin, an Arg specific ADPRTase in *Vibrio cholerae*.

Due to the great interest toward the family of ADP-ribosylating toxins, the 3D structures of several ADPRTases have been reported, including cholera toxin. Based on these known structures and the recognized sequence homologies we have predicted the 3D structure of SCO5461 using the PS2 software intended for comparative modeling of protein three-dimensional structures. Despite of the lack of extended amino acid sequence similarity, the chainfold of SCO5461 protein corresponds to the core fold of ADPRTases. In SCO5461, the conserved LYR and YVSTS motifs are part of two perpendicular β-sheets while the catalytic Glu-164 is in a loop connecting a β-sheet and an α-helix as in the ARTT loop of CT. The secondary structure of SCO5461 predicted by the SSPRED program fits to the predicted 3D

structure, since the LYR and YVSTS motifs are part of β-sheets and QVEVA is on the border of an unstructured loop and β-sheet. Whereas the third motif is EXE in Arg specific ADPRTase enzymes, as CT and MTX, QXE motifs are in toxins with different substrate specificity (Cys, Asp or DNA), therefore glutamine instead of glutamate can not determine the substrate specificity of SCO5461.

Among fully sequenced *Streptomyces* genomes the orthologues of the *SCO5461* gene were found to be present only in the closest relatives of *S. coelicolor*. All of these are hypothetic proteins (*S. lividans TK24, SSPG_02248.1*; *S. albus J1074, SSHG_04549.3; S. scabies, SCAB27771*; *S. pristinaespiralis ATCC 25486, SSDG_06196.1*, and *Streptosporangium roseum* DSM 4321, YP_003344216.1)

In the StrepDB database, SCO5461 is annotated as a putative secreted protein with possible non-cleavable N-terminal signal. The SignalP (version 3.0) program (http://cbs.dtu.dk/services/SignalP) indeed recognized a tripartite N-terminal signal sequence (1-43 amino acids) in SCO5461, which contains an RRRTAA motif in its N-terminal part that might resemble to a twin-arginine translocation (Tat) signal. We have used the prediction programs TatFind version 1.4 and TatP version 1.0 (http://www.signalfind.org and http://www.cbs.dtu.dk/services/TatP-1.0, respectively) to check whether SCO5461 protein could be a candidate Tat substrate. None of the programs recognized the N-terminal sequence of SCO5461 as a genuine Tat-targeting signal. This conclusion is in accordance with previously reported results that the SCO5461 signal peptide did not mediate the transfer of a reporter protein in *S. coelicolor* M145. Tat signal peptides superficially resemble to Sec signal peptides; however, the positively charged K41 residue at the end of the C-region of the suggested signal peptide of SCO5461 protein might function as a Sec avoidance signal, which could explain the finding that SCO5461 is not secreted in the *S. coelicolor* M145 *ΔtatC* mutant defective in Tat transport. The SCO5461 protein might be a transmembrane protein with an extracellular domain. This notion is confirmed by several protein secondary structure predictions. The SSPRED software (www.coot.embl.de/~fmilpetz/SSPRED) predicted an N-terminal α-helix which was confirmed among others by the TMpred server (http://ch.embnet.org). According to TMpred prediction, SCO5461 protein is a transmembrane protein with a short (9 aa) intracellular domain, an inside to outside transmembrane helix (26 aa) and a longer extracellular domain (27-204 aa).

4.3. Disruption of the *SCO5461* and complementation of the mutant

PCR targeted mutagenesis was used to replaced the *SCO5461* gene with an apramycin resistance cassette. Gene replacement was verified by PCR coupled to RFLP analysis of both the recombinant cosmid and the genomic DNA of the apramycin resistant *ΔSCO5461::apr* mutant. For complementation analysis, the PCR product containing the complete *SCO5461* gene was cloned into the *Eco*RI/*Hind*III site of pWHM3 creating pWHE1. The construct was transformed into the *S. coelicolor* M145 *ΔSCO5461::apr* mutant, generating the complemented *S. coelicolor* M145*ΔSCO5461::apr/*pWHE1 strain.

4.4. Phenotypic analysis of the *ΔSCO5461::apr* mutant

Morphological differentiation: Disruption of the *SCO5461* gene resulted in a conditional pleiotropic phenotype. On SFM-agar medium, the phenotype of the null mutant was similar to that of the wild type strain. Since phenotypes of mutants might depend on the growth condition, spores of wild type strain (M145), the M145 *ΔSCO5461::apr/*pWHE1 complemented mutant, and the *ΔSCO5461::apr* mutant were streaked onto the following media: SMMS, SMMS+ and R5.

On SMMS medium, the mutant showed delayed morphological differentiation compared to that of wild type M145 strain and the complemented mutant strain. In 48h, old cultures M145 and complemented mutant produced aerial mycelium and spores while the mutant had a bald-like appearance. At 120h, aerial mycelium and spore production were evident in the mutant culture as well, however, spore formation was not as dense as it was in the control culture.The applied osmotic stress in SMMS+ even more strongly delayed aerial hyphae production and sporulation. At 120h, the null mutant displayed sparse aerial mycelium and spore formation. After one week of cultivation the mutant sporulated abundantly.

However, the phenotype of the *ΔSCO5461::apr* mutant strain was different when grown on R5 medium. Both the aerial mycelium formation and sporulation were completely suppressed in the mutant growing alone on R5-agar, suggesting that normal ADP-ribosyltransferase activity is required for differentiation on this osmotically enhanced rich medium. The surface of the colonies of the *ΔSCO5461::apr* mutant had a bald-like appearance but unlike the *bldA* mutant, it produced actinorhodin. The morphological differentiation of the *ΔSCO5461::apr* mutant was restored when it was grown beside the wild

type M145 or the complemented mutant strain on R5-agar. The wild type phenotype was also restored when the mutant was cultivated on a spent R5 medium of the *S. coelicolor* M145 strain. It is important to note, that on SMMS and SMMS+ media the mutant sporulated after a longer cultivation period when it was grown in the absence of the wild type strain.

Since the complemented *S. coelicolor* M145*ΔSCO5461::apr/*pWHE1 strain had normal phenotype in all three media, we concluded that in the M145 *ΔSCO5461::apr* strain the mutant phenotype is due to the disruption of the *SCO5461* gene.

Observations of the phenotype of the null mutant strain under a phase-contrast light microscope confirmed the above-described characteristics. Phenotypes of samples were visualized by growing the cells on coverslips inserted into the agar plates. After 5 days of incubation in SMMS medium, aerial mycelium and spore chains were present in the sample of the mutant strain, but spores were less abundant than in the wild type sample. The difference between the wild type and mutant phenotypes was even more pronounced in SMMS+ medium. Beside aberrant, unevenly swollen spores, swollen hyphal tips were also frequent in the culture. The phenotype of the mutant strain grown in R5-agar plates showed, that after four days of cultivation, mostly undifferentiated mycelium and very few, short spore chains characterized the sample. Only occasional spore chains were formed even after 12 days of cultivation.

The DNA of 2 and 7 days old mycelia of wild type and null mutant strains from aforementioned three different plates with coverslips were stained with propidium-iodide. We could not find any difference in the DNA contant in the cells under confocal laser scanning microscope.

Production and secretion of antibiotic: In addition to the defect in morphological differentiation, the production and secretion of actinorhodin is also affected in the constructed null mutant. The effects of the mutation were different when the strains were cultivated on solid and on liquid media. The mutant produced and secreted more actinorhodin than the wild type strain when it was grown on R5 agar plate, and less actinorhodin was produced when it was grown on SMMS and SMMS+ agar plate. Considering the possible extracellular location of the catalytic domain of the SCO5461 protein, the differences in antibiotic secretion might confirm our previous suggestion that ADP-ribosylation of certain subunits of ABC transporter complexes might affect solute transport in *Streptomyces*. Unexpectedly the mutant did not secrete any γ-actinorhodin in liquid SFM medium, nevertheless the antibiotic production

seemed to be normal. The differences between characteristics on solid and liquid media may lie in different regulation of the antibiotic secretion.

These results suggest that some of the proteins that involved transport process of actinorhodin might be affected directly or indirectly by ADP-ribosylation that is catalyzed by the SCO5461 enzyme. None of the eight identified ADP-ribosylated proteins could be connected to the mechanism of antibiotic secretion.

4.5. Verification of ADPRTase activity of SCO5461 protein

Comparison of ADP-ribosylated protein pattern in the mutant, complemented mutant and the wild type strains evidences that *SCO5461* gene codes an ADPRTase enzyme. The absence of a 65 kDa ADP-ribosylated protein in the cytoplasmic fraction of *ΔSCO5461::apr* null mutant that was present in the sample of wild type and the complemented mutant strain confirms our hypothesis. We have identified with MALDI-TOF analysis an ADP-ribosylated protein with the same molecular mass, that protein was the solute-binding subunit of an oligopeptide ABC transporter, coded by the *SCO5477* gene. The difference in the pattern of the modified proteins of the wild type strains and the null mutant was not dramatic, which can be explained with the presence of additional ADPRTase enzyme(s) besides SCO2860 and SCO5461 proteins in the *S. coelicolor.*

5. SUMMARY

The protein ADP-ribosylation is a reversible posttranslational modification catalysed by mono-ADP-ribosyltransferases (ADPRTases). Our aim was to isolate, and identify ADP-ribosylated proteins in *Streptomyces coelicolor* A3(2), which is an antibiotic-producing bacterial strain with complex morphology, and differentiation. Also, we wanted to find new potential ADPRTase enzyme coding genes in the genome of *S. coelicolor* using sequence homology search and to prepare an ADPRTase null mutant *S. coelicolor* strain. We planned to study the biological function of protein ADP-ribosylation in *S. coelicolor* by analysing the phenotype of the null mutant strain.

First, proteins in the crude cytoplasm extract of wild type *S. coelicolor* were *in vitro* ADP-ribosylated. After optimisation of the reaction conditions ADP-ribosylated proteins were purified by m-aminophenylboronic acid affinity chromatography from crude extract of 36 hours *S. coelicolor* M145 cells cultivated on SFM medium. Samples were loaded onto 2-D SDS-PA gel. The separated proteins were in gel digested with trypsin, and the pattern of digested fragments were analysed by MALDI-TOF. Eight ADP-ribosylated proteins were identified using this method: SCO5477, SCO2198, SCO2008, SCO4771, SCO1968, SCO4824, SCO6108, and SCO7629. Six of the proteins were newly identified as ADP-ribosylated proteins, in two cases we have confirmated previous results in literature.

In silico analysis of the *S. coelicolor* genome revealed a hypothetic ADPRTase coding gene, the *SCO5461*. This *SCO5461* gene was replaced by an apramycin resistance cassette. The generated null mutant – *S. coelicolor* M145 Δ*SCO5461::apr* – strain had a pleiotropic, conditional phenotype. The mutation delayed or completely inhibited the morphological differentiation and antibiotic production and secretion of the strain, depending on culture condities. The mutant was complemented with the introduction of a wild type gene carrying plasmid. The comparison of the pattern of ADP-ribosylated proteins of the wild-type, the null mutant, and the *S. coelicolor* M145 Δ*SCO5461::apr*/pWH1 complemented null mutant strains revealed, that an ADP-ribosylated protein with a molecular weight of ~65 kDa was missing from pattern of the mutant strain. This protein, however reappeared in the complemented strain. So, we concluded that the SCO5461 is an ADPRTase enzyme which is involved in the regulation of differentiation, antibiotic production and secrecion of *S. coelicolor*.

6. PUBLICATION LIST

Publications the Ph.D. thesis is based on

Candidate: Krisztina Szirák

Neptun ID: LN1JSL

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Szirák, K.**, Keserű, J., Biró, S., Schmelczer, I., Barabás, G., Penyige, A.: Disruption of SCO5461 gene coding for a mono-ADP-ribosyltransferase enzyme produces a conditional pleiotropic phenotype affecting morphological differentiation and antibiotic production in Streptomyces coelicolor.
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Other publications

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Total IF: 8.285

Total IF (publications related to the dissertation): 2.558

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

20 November, 2012

DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA EGYETEMI ÉS NEMZETI KÖNYVTÁR

Poster and lecture related to the thesis

Szentesiné Szirák Krisztina, Keserű Judit, Schmelczer Iván, Barabás György, Biró Sándor, Penyige András (2011) Egy mono-ADP-riboziltranszferáz mutáns *Streptomyces coelicolor*. IX. Magyar Genetikai Kongresszus és XVI. Sejt- és Fejlődésbiológiai Napok, Siófok. (poster)

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