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Analysis and Identification of ADP-Ribosylated Proteins of *Streptomyces coelicolor* M145

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Mono-ADP-ribosylation is the enzymatic transfer of ADP-ribose from NAD^+ to acceptor proteins catalyzed by ADP-ribosyltransferases. Using m-aminophenylboronate affinity chromatography, 2D-gel electrophoresis, in-gel digestion and MALDI-TOF analysis we have identified eight *in vitro* ADP-ribosylated proteins in *Streptomyces coelicolor*, which can be classified into three categories: (i) secreted proteins; (ii) metabolic enzymes using NAD^+/NADH or $NADP^+$/NADPH as coenzymes; and (iii) other proteins. The secreted proteins could be classified into two functional categories: SCO2008 and SCO5477 encode members of the family of periplasmic extracellular solute-binding proteins, and SCO6108 and SCO1968 are secreted hydrolases. Dehydrogenases are encoded by SCO4824 and SCO4771. The other targets are GlnA (glutamine synthetase I., SCO2198) and SpaA (starvation-sensing protein encoded by SCO7629). SCO2008 protein and GlnA had been identified as ADP-ribosylated proteins in previous studies. With these results we provided experimental support for a previous suggestion that ADP-ribosylation may regulate membrane transport and localization of periplasmic proteins. Since ADP-ribosylation results in inactivation of the target protein, ADP-ribosylation of dehydrogenases might modulate crucial primary metabolic pathways in *Streptomyces*. Several of the proteins identified here could provide a strong connection between protein ADP-ribosylation and the regulation of morphological differentiation in *S. coelicolor*.

***Keywords*:** protein ADP-ribosylation, *S. coelicolor*, 2D-PAGE, MALDI-TOF

Streptomyces species are well known for their complex morphological differentiation process. On solid medium spores of *Streptomyces coelicolor* A3(2) 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 (Chater and Losick, 1997). Morphological differentiation process is coupled to the production of a wide variety of secondary metabolites with important medical, pharmacological, biotechnological importance (Chater, 1993, 2006).

Complete understanding of the regulation of differentiation process and antibiotic production would require the characterization of regulatory mechanisms acting on protein level. Mono-ADP-ribosylation is a post-translational protein modification, catalyzed by mono-ADP-ribosyltranferase (ADPRTase) enzymes, that transfer a single ADP-ribose moiety of β-NAD^+ to a specific amino acid side chain in a target protein. ADP-ribosylation is a reversible regulatory mechanism, it is reversed by removal of the ADP-ribose moiety by ADP-ribose-protein glycohydrolases (Hayashi and Ueda, 1985; Lowery and Ludden, 1990; Oppenheimer and Handlon, 1992; Ludden, 1994). Endogenous ADP-ribosylation of target proteins has been recognized in several organisms like the regulation of the nitrogenase enzyme in some nitrogen-fixing bacteria (Fu *et al.*, 1989; Lowery and Ludden, 1990; Ludden, 1994; Halbleib and Ludden, 2000; Yakunin and Hallenbeck, 2002), the modification of glutamine synthetase in *Rhizobium meliloti*, *S. griseus*, *Synecocystis* spp., and *Rhodospirillum rubrum* (Woehle *et al*., 1990; Penyige *et al.*, 1994; Liu and Kahn, 1995; Silmann *et al*., 1995). The presence of protein ADP-ribosylation in streptomycetes is known in *S. griseus* and *S. coelicolor* A3(2) (Penyige *et al.*, 1990, 1996; Shima *et al.*, 1996). The activity of the mono-ADPRTase enzyme changes during the life cycle; it is the highest in cells just beginning the aerial mycelium formation. The pattern of ADP-ribosylated proteins also shows characteristic changes during the life cycle (Penyige *et al.*, 1990, 1996). It is also known that the ADP-ribosylated protein pattern shows major differences in a non-sporulating mutant of *S. coelicolor* A3(2) compared to these of the wild type strain (Shima *et al.*, 1996). Recently four mono-ADP- ribosylated proteins were identified in *S. coelicolor* A3(2) (BldKB, MalE, and two periplasmic solute-binding proteins), that are extracellular, ligand-binding subunits of ABC-transporters participating in the transport of metabolites or nutrients through the membrane. These proteins are modified on a Cys residue that is part of the lipid attachment site (LTACG) of the signal peptide involved in the processing and export of these proteins to the extracel-

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lular face of the cellular membrane (Sugawara *et al.*, 2002). ADP-ribosylation of this Cys residue would very likely inhibit the proper processing of these proteins, thus inhibiting their physiological functions (Sugawara *et al.*, 2002; Piette *et al.*, 2005).

Here we summarize our work, focusing on ADP-ribosylated proteins in order to expand our knowledge about this elusive protein modification. We describe a new method based on affinity chromatography and 2D-PAGE that is suited for the isolation of proteins ADP-ribosylated not only on Cys but on any appropriate amino acid side chain. Using this method coupled to MALDI-TOF mass peptide fingerprint analysis we have identified seven new ADP-ribosylated proteins of *S. coelicolor* A3(2) M145, substantially increasing the number of known ADP-ribosylated proteins in *Streptomyces*. Our results give further support to the hypothesis that protein ADP-ribosylation could restrict the processing and transfer of membrane-anchored periplasmic proteins, and suggest possible connections between protein ADP-ribosylation and the differentiation process.

Materials and Methods

Chemicals

(Adenine-2,8-^{3}H)NAD$^+$ (1.11×10^{12} Bq/mmol) was purchased from ICN Pharmaceuticals Corp. Biochemicals (USA); immobilized pH-gradient strips, ReadyPrep™, 2D-Cleanup kit, PG buffers and chemicals necessary for 2D-electrophoresis experiments were obtained from Bio-Rad Laboratories (Germany). The m-aminophenylboronic acid-agarose for affinity chromatography was purchased from Sigma-Aldrich Co (Hungary). All other chemicals were from Sigma-Aldrich Co., unless stated otherwise.

Bacterial strains and growth conditions

S. coelicolor A3(2) M145, a plasmid-free, wild type, prototrophic, antibiotic producing strain was used in this study. Cells were cultivated on solid surface using mannitol soya fluor agar medium (SFM-agar) containing 2% (w/v) soy flour, and 2% (w/v) mannitol. A dense spore suspension [6×10^7 spores/plate in 20 mM Tris-HCl; pH 7.2 containing 0.01% (w/v) Triton X-100] was spread on the surface of cellophane-covered SFM-agar plates and cultivated at 30°C for 36 h.

ADP-ribosyltransferase assay

(Adenine-2,8-^{3}H)NAD$^+$ was used as substrate to measure ADPRTase activity as reported previously (Penyige *et al.*, 1990; Shima *et al.*, 1996). In brief, 36 h old cells were scraped off from the cellophane-covered SFM-agar plates and immediately suspended in ice-cold ADP-ribosylation buffer containing 25 mM MOPS (pH 7.4), 150 mM NaCl, 2.5 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 1.3 μl/ml protease inhibitor cocktail for bacterial extracts (P8465). Cells were disrupted by immediate sonication (Bronsonic Modell Sonifier) for 2 min with 50% cycles at 0°C. Cell debris was removed by centrifugation at 30,000×g for 20 min at 4°C and the supernatant was used in the ADPRTase assay. The supernatant was halved and one aliquot was supplemented with 500 μM ADP-ribose, the other with an equal volume of dH_2O. The reaction was started with the addition of 100 μM (adenine-2,8-^{3}H)NAD$^+$ (7.5×10^7 Bq) and the samples were incubated at 30°C. At given times 100 μl aliquots were removed from both reaction mixtures and their protein content was precipitated with 20% (w/v) trichloroacetic acid (TCA) at 0°C for 30 min. Precipitated proteins were captured on nitrocellulose membrane filters (Millipore HA, USA; 0.45 mm pore size). Filters were washed extensively with 5% (w/v) ice-cold TCA and finally with 96% (v/v) ethanol. The radioactivity remaining on the filters was determined with a liquid scintillation counter. Protein content of the sample was determined by the Bradford assay according to the manufacturer's instructions. Each assay was run in triplicates.

Purification of ADP-ribosylated proteins

Cellular crude extract was prepared as described above, except that cell debris was removed by centrifugation at 35,000×g for 30 min at 4°C. Typically mycelium from 30 plates was collected and the average protein content of the final crude extract was 8.9 mg/ml. 25 ml crude extract was incubated for 30 min at 30°C in an orbital shaker (50 rpm). After the addition of 500 μM ADP-ribose to the solution the sample was halved. One aliquot was supplemented with 200 μM NAD$^+$, the other with equal volume of dH_2O. Both samples were further incubated at 30°C for 30 min. To stop ADP-ribosylation both samples were quickly diluted into 112.5 ml ice-cold acetone and proteins were precipitated at -20°C overnight. The precipitate was collected by centrifugation at 10,000×g for 10 min at 4°C. Pelleted proteins were redissolved in 20 mM HEPES buffer (pH 8.6) containing 150 mM NaCl, 10 mM $MgCl_2$, 0.005% (w/v) sodium dodecyl sulfate (SDS) and 500 mM urea (Buffer A). The clear solution was applied to an m-aminophenylboronic acid-agarose affinity chromatography column. The column was washed with SDS and urea free Buffer A until proteins were not detectable in the flow-through by the Bradford protein assay (Bradford, 1976). Adsorbed proteins were eluted from the column with detergent-free Buffer A containing 200 mM sorbitol. The protein-containing fractions were collected and concentrated by ultrafiltration in a stirred cell [Amicon 8010] equipped with a YM 3000 filter (Millipore). The sample was further concentrated in a Centricon (Ultracell YM-3000; Millipore) filter unit. Using identical conditions, proteins of the untreated control sample were also applied and purified through the m-aminophenyl boronic acid-agarose affinity chromatography column. Concentrated proteins from both samples were separated by 2D-gel electrophoresis.

2D-gel electrophoresis of ADP-ribosylated proteins

One hundred microliters of the concentrated sample [80 μg protein/sample] was precipitated and desalted using the ReadyPrep™ 2-D Cleanup kit (Bio-Rad Laboratories) and was resolved in 125 μl rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.2% 3/10 IPG buffer, 50 mM DTT, and 0.002% (w/v) Bromophenol Blue (IEF buffer, sample buffer). For first-dimension separation isoelectric focusing (IEF) was performed using Bio-Rad Protean IEF Cell. 7 cm long pH 3~10 or 4~7 IPG strips were incubated in IEF buffer containing the sample for 16 h at room

temperature in a rehydration/equilibration tray. Thereafter proteins on the IPG strips were focused at 20°C with the following voltage gradient: from 0 to 250 V for 20 min, from 250 to 4,000 V for 2 h and 4,000 V to 10,000 Vhs. Following IEF IPG strips were processed for the second-dimension separation by a 10 min equilibration in 6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl [pH 8.8], 30% (v/v) glycerol, 1% (w/v) DTT followed by a second 10 min long bath in a similar solution in which DTT was replaced by 2.5% (w/v) iodoacetamide. The last step was immersing the strips in 1× Tris-glycine electrode buffer. The 2-D separation was performed essentially according to the method of Laemmli, in a Mini-Protean 2-D Cell (Bio-Rad Laboratories) using 5% (w/v) stacking gel and a 13% (w/v) separating gel with a constant voltage of 200 V with cooling. Three independently isolated protein samples were separated by 2D-gel electrophoresis (2D-PAGE) to obtain a representative pattern of ADP-ribosylated proteins.

In-gel trypsin digestion and MALDI-TOF identification of proteins

Coomassie-Brillant Blue stained protein spots were excised from 2D-gels. Excised pieces were destained by washing them three times with 200 mM ammonium bicarbonate, 40% (v/v) acetonitrile solution. Destained gel pieces were dried in a SpeedVac for 40 min at low temperature setting. The gel pieces were rehydrated in 25 mM ammonium bicarbonate containing 5 mM DTT for 30 min at 60°C. The pieces were then incubated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate solution for 30 min at room temperature in the dark, then washed with 25 mM ammonium bicarbonate. Proteins in gel pieces were digested by trypsin (proteomics grade Trypsin Profile IGD kit) in 40 mM ammonium bicarbonate containing 9% (v/v) acetonitrile at 37°C overnight. Digestion was terminated by the addition of 2 µl of 5% (v/v) formic acid and the extraction of peptides from the gel was enhanced by a 20 min sonication in a sonic bath. Extracted peptides were purified and desalted with a ZipTip® (Millipore) device before mixed into an appropriate α-cyano matrix solution. Samples were spotted on a MALDI-TOF sample holder plate and analyzed by MALDI-TOF mass spectrometry (Applied Biosystem Voyager-DE-STR) in a positive ion reflection mode using a pulse voltage of 20,000 V and a laser intensity of 66%. Close external calibration using standard peptides was applied. MALDI-TOF peptide mass fingerprint data were analyzed using the "MASCOT" search engine at http://matrixscience.com. Carbamidomethylation of Cys-residues was considered as a fixed modification and ADP-ribosylation was considered as the variable modification. Positive protein identification was based on a MOWSE Score better than 76. In addition, no identification was accepted unless the peptide fragment coverage was at least 30%. The sensitivity of the method is less than 50 ppm.

Results

ADP-ribosylation assay

To work out the optimal assay conditions we first analyzed the time course of protein ADP-ribosylation using (adenine-2,8-^{3}H)NAD$^+$ as substrate. Cell free crude extract was prepared from SFM-agar grown cultures of *S. coelicolor* mycelium just before the onset of aerial mycelium formation and its ADPRTase activity was determined (Fig. 1). Two parallel samples were prepared, with and without 500 µM ADP-ribose in the reaction mixture. As shown on Fig. 1, in the sample lacking ADP-ribose the ADPRTase activity peaked at 30 min, afterwards the amount of incorporated ADP-ribose decreased gradually. In the ADP-ribose supplemented sample, however, ADPRTase activity was continuously higher throughout the assay. ADP-ribose inhibits ADP-ribose-protein glycohydrolase activity (Oppenheimer *et al.*, 1992), thus stabilizing the protein-ADP-ribose complex. The addition of ADP-ribose also ensures that only enzymatic protein-ADP-ribosylation is measured in the reaction, since the "cold" ADP-ribose is in excess to [^{3}H]ADP-ribose – generated by the NAD-glycohydrolase present in the cellular crude extract from [adenine-2,8-^{3}H]NAD$^+$ – that might attach to proteins through non-enzymatic Schiff base formation (Penyige *et al.*, 1990).

Purification of ADP-ribosylated proteins

In order to understand the physiological role of protein ADP-ribosylation in *Streptomyces*, it is necessary to identify the modified proteins. We have used an aminophenylboronate affinity chromatography method to purify ADP-ribosylated proteins (Bisse and Wieland, 1992; Jobst *et al.,* 1992). For this experiment ADP-ribosylation of target proteins was carried out under modified assay conditions. First the crude extract was incubated for 30 min at 30°C to allow the enzymatic de-ADP-ribosylation of the endogenously ADP-ribosylated proteins by exploiting the presence of endogenous ADP-ribose-protein glycohydrolase activity in the crude extract. Incubation was followed by the ADP-ribosylation reaction as described in 'Materials and Methods'. ADP-ribosylated proteins from both the ADP-ribosylated and the unmodified control samples were purified then separated on a 2D gel (Fig. 2). Only those proteins were considered as ADP-

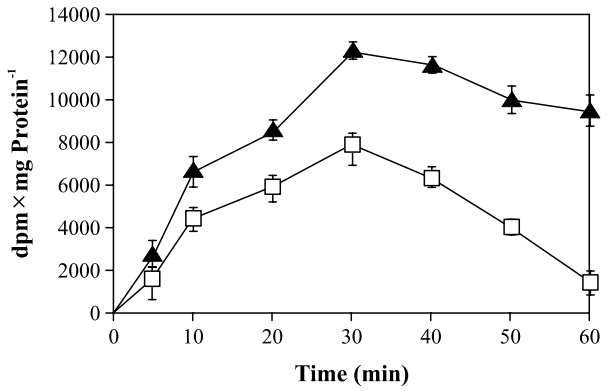

Fig. 1. Time course and the effect of "cold" ADP-ribose on protein ADP-ribosylation in *S. coelicolor* cellular crude extracts prepared from SFM-agar grown cultures. ADP-ribosyltransferase activity was determined by measuring the amount of radioactive ADP-ribose incorporated from [adenine-2,8-^{3}H]NAD$^+$ into the acid precipitable material. Each data point represents the average value (±SD) of five independent measurements. Symbols: control sample (□); ADP-ribose (500 µM) supplemented sample (▲).

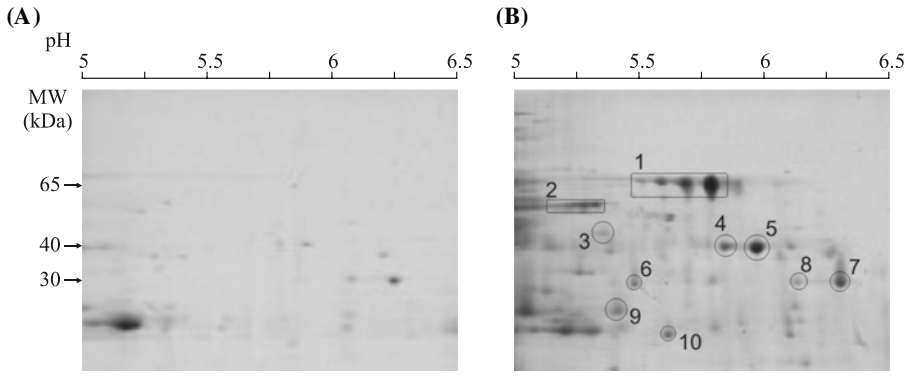

Fig. 2. The proteome map of the purified ADP-ribosylated proteins after two dimensional PAGE analysis using IPG strips pH 4.0∼7.0. The *in vitro* ADP-ribosylated proteins of total-protein extracts prepared from mycelium grown to mid-exponential growth phase were isolated by an affinity chromatography protocol using m-aminophenylboronic acid-agarose chromatography column. (A) Protein pattern of the control sample; (B) Protein pattern of ADP-ribosylated proteins. The two samples were prepared identically, except that the reaction mixture of the control sample did not contain β-NAD^+, the substrate for the ADP-ribosyltransferase enzymes.

Table 1. ADP-ribosylated *S. coelicolor* proteins identified in this study

Spot No.	Accession No. (SCODB)	Functional annotation and designation	Function category	Secretion signals	Molecular mass and pI values expected[b]/ Observed	Coverage	Match[c]
1	SCO5477	Putative oligopeptide-binding lipoprotein, bacterial extra-cellular solute-binding protein	Transport SBP[a] of ABC transporter	N-terminal signal sequence, prokaryotic membrane lipo-protein lipid attachment site	65.3 kDa ; 6.5 / 65.4 kDa; 5.8	41%	18/32
2	SCO2198	Glutamate-ammonia-ligase; Glutamine synthetase I, GlnA	Metabolism; N assimilation	None	52.6 kDa; 5.3 / 52 kDa; 5.3	55%	11/34
3	SCO2008	Putative branched chain amino acid-binding protein extracellular solute-binding protein	Transport SBP[a] of ABC transporter	N-terminal signal sequence, prokaryotic membrane lipo-protein lipid attachment site	43.5 kDa; 5.5 / 44 kDa; 5.4	47%	21/38
4 and 5	SCO4771	Putative Inosine-5'-mono-phosphate dehydrogenase	Metabolism nucleo-tide biosynthesis)	None	40.1 kDa; 5.9 / 40 kDa; 5.9 and 5.6	83%	15/41
6	SCO1968	Probable secreted hydro-lase	Metabolism (?); Glycerophosphoryl diester phosphodies-terase	Possible cleavable N-terminal region signal sequence	31.4 kDa; 5.5 / 30 kDa; 5.4	44%	6/49
7 and 8	SCO4824	FolD; Bifunctional pro-tein, Methylenetetrahydro-folate dehydrogenase/ cyclohydrolase	Metabolism; Inter-conversion of 1-C derivatives of THF	None	29.8 kDa; 6.3 / 30 kDa; 6.3 and 6.1	51%	18/35
9	SCO6108	FusH; Esterase, secreted hydrolase, fusidic acid esterase,	Fusidic acid resist-ance	Possible hydrophobic membrane spanning N-terminal domain	55.4 kDa; 5.9 / 28 kDa; 5.4	31%	12/24
10	SCO7629	SpaA; Starvation-sensing protein	Metabolism; Mande-late racemase/ muc-onate lactonizing enzyme	None	45.2 kDa; 5.9 / 24 kDa; 5.7	33%	8/22

[a] SBP: solute-binding protein
[b] matched/observed masses
[c] calculated from the sequence in the database

ribosylated that had no corresponding spot in the control sample, or those that were present in a much higher amount in the ADP-ribosylated sample. The removal of the endogenous ADP-ribose modifying groups, the *in vitro* ADP-ribosylation and the comparison of the 2D gels would allow us to distinguish ADP-ribosylated proteins from glycosylated proteins that might be present in the cytoplasmic extract of *S. coelicolor.* Although the presence of cell-associated or secreted prokaryotic glycoproteins has been recognized recently, no glycosylated proteins have been reported in *Streptomyces* (Messner, 1997). The inclusion of SDS and urea in the binding buffer helps to avoid co-purification of protein associated with the ADP-ribosylated proteins.

It is possible that endogenously ADP-ribosylated proteins still could be present in the control, since ADP-ribosylated proteins with a slow turn-over rate for de-ADP-ribosylation would bind to the column in control samples too.

Identification of ADP-ribosylated proteins

The identities of the proteins represented by the Coomassie Blue-stained spots on the 2 D gel were determined by MALDI-TOF analysis of their in-gel digested tryptic fragments. The analyzed spots (Fig. 2B) corresponded to 8 different proteins (Table 1).

Spot 1. Isoforms of the putative oligopeptide-binding lipoprotein (an ABC transporter subunit) encoded by SCO5477 were identified in all spots of the framed spot 1. SCO5477 is a paralogue of BldKB, it also contains a membrane lipoprotein lipid-attachment site (LLTTAACG) and is expected to be a periplasmic, membrane-attached protein. Our MALDI-TOF data showed the presence of the tryptic peptide 1-9 (MTTQRTSGR) at the expected mass of 1037.5 showing that the protein is an unprocessed full-length protein. The position of all isoforms corresponded to the expected molecular mass of the full protein, though their pIs are more acidic than the expected value (Table 1).

Spot 2. Isoforms of glutamine synthetase I (GSI, GlnA, SCO2198; glutamate-ammonia-ligase) were present in this spot. The major isoform in spot 2 had the molecular mass and pI value that corresponded to the expected position of *S. coelicolor* GSI. All isoforms in spot 2 gave a tryptic peptide at 2807.4 Da corresponding to the peptide 395-420 containing the conserved Tyr-397 residue indicating that the protein was not adenylylated.

Spot 3. The putative periplasmic branched-chain amino acid-binding protein SCO2008 was identified in this spot. The same protein was found to be ADP-ribosylated on a Cys residue that is part of the signal sequence and actually the lipid attachment site of this extracellular solute-binding protein in a previous study (Sugawara *et al*., 2002).

Spots 4 and 5. The putative inosine-5'-monophosphate dehydrogenase (IMPDH) was identified in spots 4 and 5. The position of spot 5 corresponded to the position of an unmodified IMPDH, the isoform in spot 4 showed similar molecular mass but its pI is more acidic. Interestingly, the analysis of the MALDI-TOF peptide mass fingerprint data with the optional modification extension in "MASCOT" search engine provided evidence about the specific nature of the modification. Our data showed the presence of tryptic peptides 207-225 and 226-245 at the expected mass of 1699.8 Da and 2123.1 Da in the major protein spot, however, in the isoform in spot 4 the molecular mass of the same fragments increased by 541 Da (to 2240.8 and 2664.1, respectively), corresponding to the addition of ADP-ribose. Analysis of the amino acid sequence data suggested that IMPDH was modified at two different positions, at Arg-225 and Arg-244. These adjoining fragments 207-225 and 226-245 are parts of a highly conserved region of the IMPDH enzyme. These are shown in Fig. 3 as a representative for the mass spectra analysis.

Spot 6. Again a novel ADP-ribosylated protein, SCO1968, a putative secreted hydrolase (glycerophosphoryl diester pho-

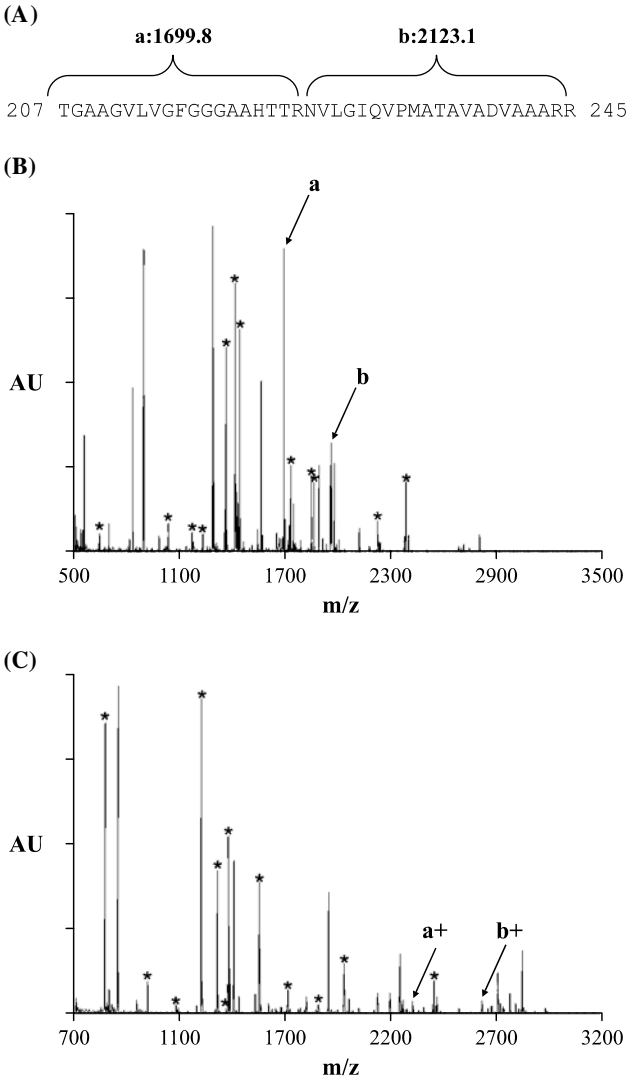

Fig. 3. MALDI-TOF peptide mass fingerprint analysis of tryptic digests of SCO4771 (IMPDH) protein spots excised from the 2D gel. (A) Amino acid sequence of IMPDH showing the peptides recognized as ADP-ribosylated fragments. (B) Peptide mass fingerprint of unmodified IMPDH. Peaks annotated as a and b correspond to the annotated peptides in (A). (C) Peptide mass fingerprint of ADP-ribosylated IMPDH. Peaks annotated as a+ and b+ correspond to a + ADP ribose (i.e. +541.0 Da) and b + ADP-ribose (i.e. +541.0 Da). * labels those peptides that were identified.

sphodiesterase family protein) was identified in this spot. The pI of the protein according to its position on the 2D gel is more acidic than that of the unmodified form and the molecular mass is slightly lower than the expected mass. The observable net gain in negative charge might reflect ADP-ribosylation [ADPR typically produces a ΔpI of 0.1∼0.2 (Vincent *et al.*, 1999)] and the slightly increased electrophoretic mobility on the SDS gel could also be the result of ADP-ribosylation (Bornancin *et al.*, 1992). Such a shift in electrophoretic mobility is not always observable or even opposite changes in mobility had also been described for ADP-ribosylated GTP-binding proteins (Bornancin *et al.*, 1992; Vincent *et al.*, 1999). We were not able to identify the modified fragment or amino acid.

Spot 7 and 8. The bifunctional protein FolD (methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase; SCO4824) was identified in these spots. Spot 7 is found in the correct position of FolD, therefore this has to be the unmodified protein. Spot 8, however, had a more acidic pI than expected. The tryptic digest fingerprint of Spot 8 revealed the absence of the peptide fragment 158-166 (885.5 Da), and the presence of an unexpected fragment of 1426.4 Da. The mass difference is 541, corresponding to the molecular mass of ADP-ribose. This fragment contains Arg166, a possible target for arginine-specific ADP-ribosylation (data not shown).

Spot 9. The protein in this spot is FusH (SCO6108) a secretable esterase, a novel ADPRTase target. It is known that *fusH* codes for a highly specific esterase that confers resistance against fusidic acid (Haar *et al.*, 1997). Spot 9 had a much lower molecular mass and more acidic pI than those of the unmodified protein. It is possible that this spot contained only a fragment of the enzyme, since this protein is produced as a zymogen precursor that is cleaved by limited proteolysis to generate the active form.

Spot 10. This is also a novel ADP-ribosylated protein, SCO7629 or SpaA belonging to the mandelate racemase/muconate lactonizing enzyme family, homologous to the starvation phase regulating protein RspA of *Escherichia coli* (Huisman and Kolter, 1994; Piette *et al.*, 2005). The site of modification was not identified in this protein, since tryptic fragments of the spot represented only the C-terminal half of the protein.

Discussion

Our results from the time course of protein ADP-ribosylation in *S. coelicolor* (Fig. 1) were consistent with the presence of a reversible protein ADP-ribosylation/de-ADP-ribosylation cycle. The loss of radioactivity from proteins was very likely due to enzymatic de-ADP-ribosylation possibly carried out by protein-ADP-ribose glycohydrolase since this reaction is inhibited by an excess of ADP-ribose, the end product of the reaction (Oppenheimer and Handlon, 1992). This notion is supported by the fact that the *S. coelicolor* genome contains at least six genes whose products show considerable sequence homology to known protein-ADP-ribose glycohydrolase enzymes (SCO0086, SCO1766, SCO2028, SCO2030, SCO4435, and SCO5809).

To identify actual ADP-ribosylated proteins we have used m-aminophenylboronate affinity chromatography, 2D-gel electrophoresis, in-gel digestion and MALDI-TOF analysis. We have to note that several proteins are represented by more than one spot on the 2D gel. These proteins could be classified into two groups. In one group (represented by the framed spots) beside the major spot there are several isoforms of the same protein with the expected molecular mass but with slightly different pI values. These isoforms may result from post-translational protein modification or spontaneous deamidation of Asn and Gln residues explaining the shift toward more acidic pI (Zomber *et al.*, 2005). The other group consists of proteins that have only two forms, one in the expected position and a second which has identical molecular mass but different pI (spot pairs: 4 and 5; 7 and 8). In all these cases the irregular spots had more acidic pI than expected and ADP-ribosylation (through the addition of two phosphoric acid residue) is expected to lower the pI of proteins, since a slight shift towards the acidic side of gel is known to occur when a protein becomes phosphorylated or ADP-ribosylated (Rieul *et al.*, 1987).

Our study expanded the current knowledge about protein ADP-ribosylation and complements the previous proteomic analysis (Sugawara *et al.*, 2002). On the 2D gel eight proteins were identified unambiguously, belonging to three classes; (i): secreted proteins; (ii) dehydrogenase enzymes, and (iii) other proteins.

The four secreted proteins could be classified into two functional categories. SCO2008 and SCO5477 are members of the family of extracellular solute binding proteins. In a previous study Ochi and co-workers identified SCO2008 as an ADP-ribosylated protein (Sugawara *et al.*, 2002). The site of ADP-ribosylation is identified to be the conserved Cys residue in the consensus cleavage site sequence, part of the N-terminal motif that mediates surface anchoring. Based on the presence of the same Cys containing motif SCO5477 was suggested as a possible target for ADP-ribosylation in another study (Piette *et al.*, 2005). Identification of the SCO5477 protein in our sample supports this hypothesis. In the same study the authors hypothesize that ADP-ribosylation of the Cys in the signal sequence motif might interfere with the transport and periplasmic localization of these ligand-binding proteins.

The other two secreted proteins, encoded by SCO6108 (FusH) and SCO1968, are hydrolases. FusH is a resistance factor, it is a highly specific esterase that deacetylates and thus inactivates the steroid antibiotic fusidic acid (Haar *et al.*, 1997). The cytoplasmic localization of FusH in our sample might suggest that ADP-ribosylation of the protein could interfere with its transport to the extracellular space. SCO 1968 is a putative secreted hydrolase, similar to the *B. subtilis* GlpQ, a phosphate starvation-induced glycerophosphoryl diester phosphodiesterase which is involved in the hydrolysis of deacylated phospholipids to glycerol-3-phosphate (Antelmann *et al.*, 2000). ADP-ribosylation of this enzyme could be an additional post-translational regulatory mechanism to control glycerol utilization in *S. coelicolor.*

The second group of ADP-ribosylated proteins contains SCO4771, a putative IMPDH of *S. coelicolor*. Since the intracellular GTP plays a crucial role in the regulation of the morphological differentiation in prokaryotes and IMP-dehy-

drogenases catalyze the rate-limiting reaction of *de-novo* GTP biosynthesis, ADP-ribosylation of IMPDH might be an important physiological regulatory mechanism in spore and antibiotic production (Ochi, 1987; Inaoka and Ochi, 2002). The other protein in this group is FolD, encoded by SCO4824. This is a bifunctional enzyme with methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activity, involved in the metabolism of tetrahydrofolate. The suggested modified peptide fragment GAEVVVVGR is part of the highly conserved $NADP^+$-binding motif (Allaire *et al*., 1998). Since ADP-ribosylation often results in inactivation of the target protein, ADP-ribosylation of this enzyme might modulate crucial primary metabolic pathways or even differentiation by interfering with methionine and purine biosynthesis.

For the IMPDH and FolD proteins the MASCOT program with optional extension for protein modifications recognized the ADP-ribosylated peptide fragments, though an MS/MS identification would make the data more reliable. According to a recently published method, ADP-ribosylated peptides and the modified amino acid in the peptide could be identified in a single LC-MS/MS experiment (Hengel *et al*., 2009). In the paper the authors demonstrated, that collision-induced dissociation and infrared multiphoton dissociation fragmentation techniques followed by ECD analysis is the best method to identify unambiguously the site of ADP-ribosylation in a peptide.

The third group contains two proteins. The starvation sensing protein SpaA (encoded by SCO7629) is homologous to the stationary phase regulatory protein RspA of *E. coli* (Huisman and Kolter, 1994). In a previous study disruption of this gene shows a conditional cell density dependent mutant phenotype, causing delayed antibiotic production and increased actinorhodin production in high cell density cultures in a poor culture medium (Schneider and Chater, 1996). Endogenous ADP-ribosylation of this enzyme might affect the cellular response to cell density. GlnA (SCO2198) is the other protein of this group, its activity is known to be regulated by post-translational adenylylation in prokaryotes (Lowery and Ludden, 1990; Woehle *et al.*, 1990) and GlnA is found to be adenylylated on a conserved Tyr-397 residue in *S. coelicolor* (Hesketh *et al.*, 2002). GlnA, however, is also shown to be ADP-ribosylated in several bacterial species, like in *S. griseus*, *Synecocystis* spp., and *Rhodospirillium rubrum* (Woehle *et al.*, 1990; Penyige *et al.*, 1994; Silmann *et al*., 1995). The results presented here confirm our previous findings about the *in vitro* ADP-ribosylation of this enzyme in *Streptomyces* (Penyige *et al.*, 1994).

In case of proteins SCO4771 and SCO4824 both the unmodified and modified forms of the proteins were found on the 2D gel. The similarity between these proteins is that both of them exert their physiological function as a multimer in the cell. One might suggest, that the activity of the multimer depends on the ratio of unmodified and ADP-ribosylated subunits in the complex. This would allow a fine-tuning of the activity of the whole functional unit according to the metabolical needs of the cell.

While protein ADP-ribosyaltion has been recognized several years ago in *Streptomyces*, we still do not have much information about the ADPRTase enzymes present in these organisms. From the results published by Sugawara *et al*. (2002), it is very likely that *S. coelicolor* harbors Cys-specific ADPRTase activity. It is also known, that *S. coelicolor* possesses the *arr* (SCO2860) gene that is highly similar to *Mycobacterium smegmatis* rifampin ADP-ribosyl transferase. Using PSI-BLAST search we have found the SCO5461 gene that contains the consensus motifs that form the NAD^+-binding site (Domenghini *et al*., 1991). The closest homologues of this gene are the pierisins (apoptosis-inducing toxins of the cabbage butterfly) and the *Bacillus sphaericus* LP1-G mosquitocidal toxin, both proteins are confirmed ADPRTase enzymes (Takamura-Enya *et al.*, 2001). The mutational analysis of the SCO5461 is underway in our laboratory.

In conclusion, the study presented here identified eight ADP-ribosylated proteins in *S. coelicolor*. Seven of them are novel targets for ADP-ribosylation and in two cases we were able to confirm previous results. In one case (SCO5477) we provided experimental support for the general predicament of ADP-ribosylation as a possible mechanism to regulate membrane transport and localization of periplasmic proteins (Piette *et al.*, 2005). It is also noteworthy, that several of the proteins identified here - SCO5477; SCO4771, IMPDH; SCO7629, SpaA; SCO4824, FolD - could provide a connection between protein ADP-ribosylation and morphological differentiation, a prediction we have reported previously (Penyige *et al*., 1990, 1996; Shima *et al*., 1996).

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