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PROTEOMIC INSIGHT INTO THE PRIMYCIN FERMENTATION PROCESS OF *SACCHAROMONOSPORA AZUREA*

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Saccharomonospora azurea SZMC 14600 is a member of the family Pseudonocardiaceae exclusively used for industrial scale production of primycin a large 36-membered non-polyene macrolide lactone antibiotic belonging to the polyketide class of natural products. Even though maximum antibiotic yield has been achieved by empirically optimized two-step fermentation process, little is known about the molecular components and mechanisms underlying the efficient antibiotic production. In order to identify differentially expressed proteins (DEPs) between the pre- and main-fermentation stages of primycin, comparative 2D-PAGE experiments were performed. In total, 98 DEP spots were reproducibly detected, out of which four spots were excised from gels, and identified through MALDI-TOF/TOF mass spectrometry. Peptide mass fingerprint analysis revealed peptide matches to HicB antitoxin for the HicAB toxin-antitoxin system (EHK86651), to a nucleoside diphosphate kinase regulator ((Ndk; EHK81899) and two other proteins with unknown function (EHK88946 and EHK86777).

Keywords: Differentially expressed proteins – HicB-family protein – primycin – *Saccharomonospora azurea* – two-dimensional protein gel electrophoresis

INTRODUCTION

As the problem of antimicrobial resistance becomes more widespread, the need for new anti-infective agents is more urgent than ever [20]. Actinomycetes are known as one of the most significant producers of pharmacologically important metabolites with over 10,000 bioactive compounds identified [1, 15]. Our previous study demonstrated that *Saccharomonospora azurea* SZMC 14600 strain has an enhanced ability to produce primycin, a non-polyene macrolide lactone antibiotic [11]. This heat stable, organic solvent soluble antibiotic complex (A1, A2 and C1) was first described by Vályi-Nagy et al. [18]. Primycin (primycin sulphate) is an active ingredient of Ebrimycin gel that has been successfully applied to prevent the bacterial infection of surface traumas and burned tissues. Comparative *in vitro* re-investigation of the efficacy of primycin clearly demonstrated that the antibiotic possesses high activity against the most frequent Gram-positive pathogens including some multi-drug resist-

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ant strains, without remarkable resistance development [8]. To get a deeper insight into the bioactive natural products metabolism of *S. azurea* SZMC 14600 whole-genome sequencing was performed [5]. The genome project clearly demonstrated that *S. azurea* is a prolific source of structurally diverse secondary metabolites, however, the *in silico* information was not tied to products in the laboratory until now. In order to further support our structural genomic data, in this pilot study a proteomics approach was performed. Two-dimensional protein gel electrophoresis (2-DE) followed by MALDI-TOF mass spectrometry supported by bioinformatics tools allowed in-depth-analysis for understanding complex biological process of microbial secondary metabolism [4]. Our preliminary comparative proteomic study of *S. azurea* cultivated sequentially in pre- and main-fermentation medium revealed considerable quantitative and qualitative differences in protein profiles. Among the differentially expressed and clearly detectable proteins a nucleoside diphosphate kinase regulator, one of the HicB-family proteins, and two proteins with unknown functions were identified.

MATERIALS AND METHODS

Saccharomonospora azurea SZMC 14600 freeze-dried stock cultures maintained at –80 °C in Luria broth (Sigma-Aldrich) with 20% glycerol were used to directly inoculate 50 mL of pre-fermentation medium (PF) containing 3% (w/v) soy flour; 4.2% (w/v) water soluble starch; 0.36% (w/v) NaCl; 0.6% (w/v) $CaCO_3$ and 0.5% (w/v) sun oil (pH 8). PF-culture was grown for 2 days at 37 °C in an orbital shaker at 200 rpm. Thereafter 1 mL suspension of bacterial cells was used to inoculate 35 mL of main-fermentation medium (MF) containing 4% (w/v) soy flour; 4% (w/v) water soluble starch; 0.3% (w/v) NaCl; 0.5% (w/v) $CaCO_3$; 0.6% (w/v) sun oil; 0.3% (w/v) stearic acid and 0.1% (w/v) KH_2PO_4 (pH 9.5). The submerged MF cultures were incubated for 5 days on a rotary shaker (200 rpm) at 28 °C.

Soluble proteins were extracted from 300 mg fresh weight of mycelia mats as described by Wang et al. [19]. Upon completion of the first dimension, samples containing 250 µg of total protein were mixed with rehydration buffer. Isoelectric focusing of rehydrated protein samples were performed on 7 cm IPG Strips (pH 3–11, Bio-Rad) at 250 V for 15 min (rapid voltage ramping), at 4000 V for 1 hour (linear voltage ramping), at 4000 V for 15 hours (rapid voltage ramping). Finally, in order to avoid potential artefacts and to prevent diffusion of focused proteins, a hold step was maintained at 500 V until the run was stopped. After focusing, the IPG strips were equilibrated in 6 M urea; 20% (v/v) glycerol; 2% (w/v) SDS; 0.05 M Tris-HCl (pH 8.8), and 2% (w/v) DTT for 20 min. The second dimension protein separations were performed on 12.5% (w/v) sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels, stained with Coomassie Brilliant Blue (Sigma-Aldrich). Gel images were captured by AlphaImager high performance gel documentation system (Protein Simple, Alpha Innotech Co. San Leandro, CA) and protein spots were quantified using Prodigy SameSpots 2D software-package (Xpedition version 1.0, Alpha Innotech)

according to the manufacturer's instructions. The normalized volume intensity was used for comparison between groups. The spots whose normalized volume intensity was higher than 1.5 and lower than 0.5 with respect to the control (LB or EF) at the level of $p < 0,05$ were defined as differential expressed protein (DEP). Three independent biological replicates were performed under each condition (cultivation on LB, EF and MF).

Four of the differentially expressed protein spots were excised from the gel and destained by washing three times for 10 min in 200 µL of 50% (v/v) acetonitrile solution containing 50 mM NH_4HCO_3. The disulphide bonds of cysteines were reduced by 50 µL of 20 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 for 1 h at 55 °C. The alkylation of the cysteine groups was carried out in 50 µL of 20 mM iodoacetamide solution. The gel pieces were dehydrated at room temperature by a Speed Vac Concentrator (Speed Vac Plus, SC100A, Savant) and proteins were in-gel-digested in 10 µL of 40 µg/µL modified trypsin (Promega, Madison, WI) in Tris buffer (2.5 mM, pH 8.5) at 37 °C overnight [16]. The digestion reactions were stopped with 15 µL aqueous solution of acetonitrile and formic acid (49/50/1 v/v/v).

Protein samples were analysed using a MALDI TOF/TOF MS (Bruker Daltonics). Proteins from MS/MS spectra were identified using ProteinScape 2.1 server utilizes the MASCOT PMF database search software (www.matrixscience.com., Matrix Science Ltd.) and Bruker BioTools 3.2 software (Bruker Daltonics) accessing the MSDB, Swiss-Prot and NCBI non-redundant protein databases. The search parameters were set as follows. Two missed cleavages were allowed; carbamidomethyl was set as fixed and oxidized methionine as variable modification, monoisotopic peptide masses was set to 150 ppm. In parallel, to improve the coverage and confidence of identified peptides open reading frame database of *S. azurea* SZMC14600 was also applied. Virtual protein patterns corresponding to the genomic data were generated by JVirGel 2.0 software [10]. All experiments were performed in triplicate.

RESULTS

Proteomics techniques based on two-dimensional gel electrophoresis are capable of simultaneously separating thousands of proteins from a single organism. Our comparative whole genome analysis of *S. azurea* SZMC14600 revealed 4554 potential protein coding genes, among them 209 (4.5%) were considered involved in the regulation of secondary metabolism, transport and metabolic processes (unpublished data). The comparison of the protein profiles of *S. azurea* cells obtained from the sequential culturing process in LB, PF and MF media revealed on average 402 ± 23, 422 ± 31 and 424 ± 33 detectable protein spots, respectively (Fig. 1). The finding that a vast majority of proteins were separated in the molecular mass range of 10–88 kDa and had a pI range of 4.0–7.0 is in good agreement with previously published data [17, 21]. Among the visualized proteins $77 \pm 12\%$ were consistently present on all gels, nevertheless changing culture media from LB to PF and subsequently from PF to MF revealed 39 and 43 newly appeared (up-regulated) protein spots, respectively

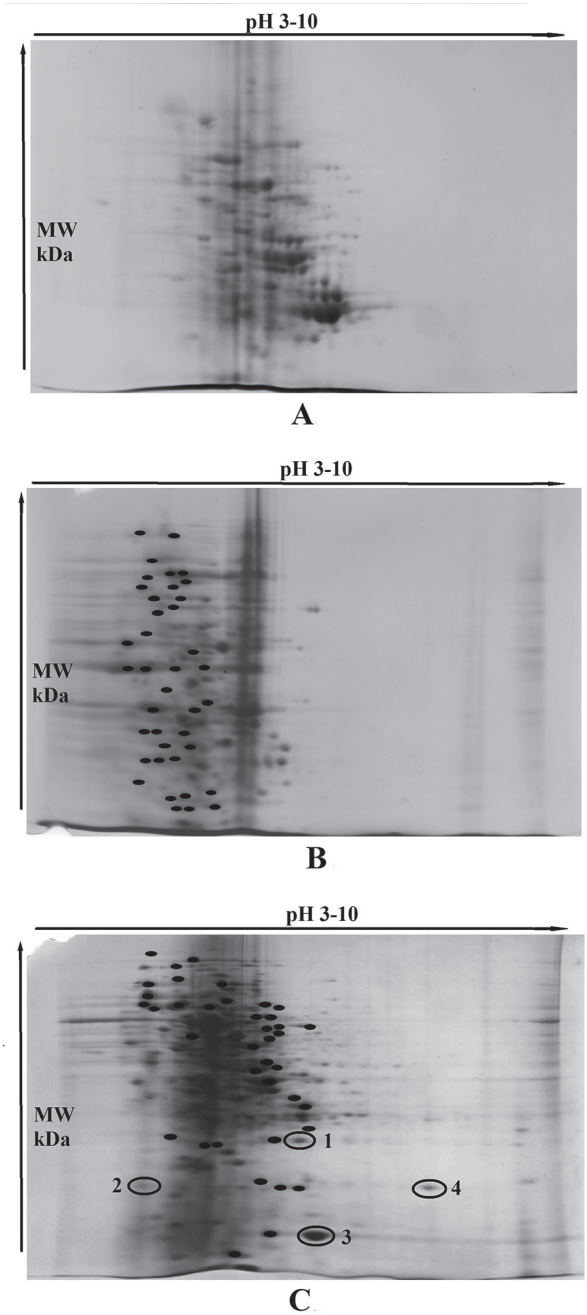

Fig. 1. Two-dimensional gel electrophoresis images of the total proteins from high primycin producer *S. azurea* SZMC 14,600 grown in: A) LB medium, B) Pre-fermentation medium (PF), C) Main-fermentation medium (MF). The presence of newly appeared protein spots, which were detectable in all gels are indicated by small black dots (B, C). Individual peptide spots (C) are numbered accordingly: 1. HicB-family protein; 2. Nucleoside diphosphate kinase regulator; 3. and 4., unknown proteins

Table 1
List of the identified proteins corresponding to excised spots from 2-DE by MALDI TOF/TOF MS system

Spot' number	Identified protein	Protein ID	Score	Calculated MW (Da) pI	Fold change MF/PF	P value
1	HicB-family protein	EHK86651	107	19158 – 6.54	11.5±2.3	0.009
2	Nucleoside diphosphate kinase regulator	EHK81899	46	16000 – 5.13	6.3±1.1	0.036
3	Unknown protein	EHK88946	70	15933 – 6.80	13.8±0.8	<0.001
4	Unknown protein	EHK86777	106	18065 – 8.19	14.3±0.7	<0.001

(Fig. 1B, C). In this pilot study, four of the MF medium induced peptide spots (Fig. 1C), that may be involved in enhancing the production of primycin were excised and analysed using MALDI TOF mass spectrometry. The corresponding single charged monoisotopic peptide masses were searched against MSDB, Swiss-Prot and NCBInr databases and revealed significant homology with one of the HicB family proteins, a nucleoside diphosphate kinase (Ndk) regulator and two other unknown proteins (Table 1). The most exciting and unexpected finding was the appearance of a HicB protein (antitoxin for the HicAB toxin-antitoxin system) in parallel with the enhanced primycin synthesis during the second phase of fermentation process.

DISCUSSION

It is widely accepted that bacterial secondary metabolism usually starts in the stationary phase of cell growth (idiophase) when cells encounter adverse environmental conditions, as represented by depletion of essential nutrients or the presence of different stress stimuli. Genomics-based technologies, including whole genome sequencing, transcriptome, proteome, and metabolome profiling, together with *in silico* modelling and simulation, have become vital tools in the recently evolved industrial system biology [13].

Since the first description of plasmid encoded bacterial toxin-antitoxin (TA) systems [14], several chromosomally located TA systems have been studied, however, their biological functions are still under debate [6, 12]. Chromosomal TA systems are small genetic modules classified into five types depending on the nature and mode of action of the antitoxin [9]. Although type II TA systems are probably the most abundant and the best described class of TA systems, little is known about the roles of HicAB protein homologs of Gram-positive bacteria [2, 7]. In type II class, the antitoxin is a small protein capable of blocking the toxin's disruptive behaviour by direct protein binding or blocking the promoter region of the TA operon. Our finding that HicB antitoxin was detected exclusively in late stage of the antibiotic fermentation

process underscore the hypothesis that TA systems could play a role in secondary metabolite production activated by nutrient starvation or other forms of environmental stresses.

Additionally, three differential protein spots were found related to the main-fermentation step (Table 1). Spots 3 and 4 (Fig. 1B) represent predicted proteins with unknown functions, while the spot 2 corresponds with the nucleoside diphosphate kinase regulator protein. Ndk is a major housekeeping enzyme in the production of NTPs and dNTPs that are fundamental for DNA/RNA synthesis, cell division, macromolecular metabolism and growth, thus Ndk is important in the regulation of stationary phase survival of the bacterial cells [3].

Taken together, according to our best knowledge, this is the first report which offers proteomic insight into the primycin fermentation process of *S. azurea* SZMC 14600.

As a first step to create an optimal workflow for large-scale experiments, methods of high-resolution protein separation and parameters for peptide mass fingerprinting identifies proteins were optimized. Our pilot study based on SDS-PAGE followed by MALDI-TOF mass spectrometry revealed significant differences in protein profiles between the two stages of primycin fermentation process, however, further studies are required to clarify the potential role of these proteins.

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