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From Microbial Proteomics to Synthetic Biology: *Amycolatopsis balhimycina* case

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Actinomycetes, filamentous Gram-positive bacteria, are usually exploited as bio-farms naturally producing a wide range of small biologically active metabolites, such as antibiotics, extensively used in medicine, food-industry, chemistry and bio-remediation strategies. The development of high throughput technologies, like proteomics, allows functional genomic studies aimed at shedding light on molecular mechanisms controlling the production of useful compounds and macromolecules. Differential proteomic analyses, performed by using Two Dimensional PolyAcrylamide Gel Electrophoresis (2D-PAGE) coupled to mass spectrometry (MS) procedures, revealed novel links between balhimycin production (a vancomycin-like antibiotic) and metabolic pathway regulation in *Amycolatopsis balhimycina* DSM5908. In particular, our investigation, performed by combining data from differential proteomic analyses carried-out using wild-type (Wt) and non-producing strains incubated in different growth conditions, showed that antibiotic production is always associated with the up-regulation of either specific enzymes of balhimycin (*bal*) biosynthetic gene cluster and enzymes related to central carbon metabolism, cell energy and redox balance. Thus this approach suggested new insights to improve fermentation technology strategies and revealed target genes for synthetic biology approaches aimed to improve antibiotic yield production.

1. Introduction

Actinomycetes, filamentous Gram-positive bacteria, are usually exploited as bio-farms naturally producing a wide range of small bio-active metabolites used in medicine, food-industry, chemistry and bio-remediation strategies (van Wezel and McDowall, 2011). Bio-active metabolites are known to be synthesized by producing strains as powerful molecular weapons to be used in the challenge for nutrients. Environmental conditions and stimuli thereof are signals promoting the synthesis of these compounds through the activation of a cascade of regulators controlling the expression of dedicated genes.
Active metabolites like antibiotics are generally synthesized at very low yields. Therefore, rational genetic engineering strategies aimed at increasing product yields are of interest and could take advantage from global gene expression analysis. In fact, the development of high-throughput techniques to study global gene expression demonstrated new opportunities to understand the relationships between basal metabolism and bio-active molecules synthesis. In particular Differential proteomic analyses, performed by using Two Dimensional PolyAcrylamide Gel Electrophoresis (2D-PAGE) coupled to mass spectrometry (MS) procedures, are valuable tools to study gene expression

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profile changes during growth, generally portrayed as sets of genes being up- or down-regulated. These changes give clues to the regulation of many biological processes and metabolic pathways that support either biomass and antibiotic production.

The actinomycete *Amycolatopsis balhimycina*, which produces the vancomycin-like antibiotic balhimycin (Pelzer et al., 1999), was investigated as model strain for studies on glycopeptide biosynthesis since it can be genetically modified and the sequence of *bal* cluster, containing genes devoted to balhimycin biosynthesis, is available. Balhimycin consists of a heptapeptide core made of the proteinogenic amino acids Leu and Asn and the nonproteinogenic amino acids 3,5-dihydroxyphenylglycine (DPG), 4-hydroxyphenylglycine (HPG) and β-hydroxytyrosine (H-Tyr). This heptapeptide is assembled by a non-ribosomal peptide synthetase (NRPS) and then extensively modified by the so called tailoring reactions, such as oxidative cross-linking of the electron-rich aromatic side chains, halogenation, glycosylation and methylation (Süssmuth and Wohlleben, 2004).

Recently, proteomic investigations on *A. balhimycina* were carried out to study relationships between global protein expression profiles and antibiotic production. In particular, comparative analyses were carried out:

1) before and during balhimycin producing growth stages in the wild-type (Wt) strain incubated in flask (Gallo et al., 2010a);
2) between Wt and the two non-producing strains *A. balhimycina* SP1-1 and Δ*oxyD*, incubated in flask (Gallo et al., 2010a);
3) between producing and non-producing conditions achieved by using Wt strain chemostat cultivations (Gallo et al., 2010b).

All these investigations revealed correlation between balhimycin production and the up-regulation of enzymes required for production energy and metabolic intermediates necessary for balhimycin building block synthesis. In the present work, these data are combined to unravel common regulatory and metabolic inferences necessary for the production of balhimycin.

2. Methods

2.1 Bacterial strains

A. balhimycina DSM5908 (WT), *A. balhimycina* SP1-1 (*oxyA*::pSP1.5Pst::*oxyB*) (Pelzer et al., 1999) and *A. balhimycina* OP090 (Δ*oxyD*) (Puk et al., 2004) strains were a gift of Prof. Wohlleben, University of Tubingen, Germany.

2.2 Culture conditions in flask and chemostat fermentations

A. balhimycina biomass samples harvested for proteomics from flask cultivations were obtained as described in Gallo et al., 2010a. In particular, a chemically defined medium, named MG, containing maltose as carbon source and glutamate as carbon and nitrogen sources was used.

A. balhimycina biomass samples harvested for proteomics from chemostat experiments were obtained as described in Gallo et al., 2010b. In particular, two defined media, LP and LG, having the same composition with the exception of glucose and inorganic phosphate concentrations (12 g/l and 0.6 mM in LP, respectively; 6 g/l and 1.8 mM in LG, respectively).

Balhimycin production was monitored using microbiological assay and/or HPLC analysis as described Gallo et al., 2010a and 2010b.

2.3 Total protein extraction

Total protein content was extracted from harvested mycelia as described in Gallo et al., 2010a and 2010b.

In particular, after washing steps in buffer (W) containing a cocktail of protease inhibitors, cells were broken by sonication in the W buffer added with 3% SDS to solubilize proteins. Protein extraction was placed in boiling water for 5 min. for denaturing and then cooled on ice for 10 min. DNAse (100 µg/ml) and RNAse (50 µg/ml) mix was added to protein extractions to remove nucleic acid interfering with successive protein isoelectrophocusing (IEF). After dialysis against distilled water at 4°C, and acetone precipitation at −20°C, proteins were suspended in IEF buffer as described in Gallo et al., 2010a and 2010b.

2.4 Generation of 2D-protein maps and protein identification

For proteins separation by IEF (firs dimension) and SDS-PAGE (second dimension) general guidelines from 2-D Electrophoresis manual (Berkelman and Stenstedt,1998) and from Ettan DIGE System user manual (GE Healcare) were followed with modifications as described in Gallo et al., 2010a and 2010b. *Amycolatopsis balhimycina* 2D protein maps are also available in the interactive web site at address http://www.unipa.it/ampuglia/Abal-proteome-maps.

2D-proein map images were analyzed in silico for differentially abundant proteins by using ImageMaster 2D platinum software 5 and successive versions.

Differentially abundant protein spots were excided by 2D-gels and identified by mass spectrometry procedures as described in Gallo et al., 2010a and 2010b.

3. Results and Discussion

3.1 Balhimycin-controlled protein expression

Recently, comparative proteome analyses revealed 73 protein spots having a balhimycin- dependent expression. In fact, these proteins were differentially abundant before and during antibiotic production in *A. balhimycina* Wt flask cultivations and also differentially abundant in two *A. balhimycina* non-producing strains in respect to Wt one in producing growth stages (Gallo et. al, 2010a). Interestingly, these proteins were involved in central carbon metabolism, including glycolysis/gluconeogenesis, tricarboxylic acid (TCA) cycle, pentose phosphate (PP) pathway, cell energy and redox balance and in the biosynthesis of protein, balhimycin and cofactor (Figure 1).

The expression of these proteins was also analyzed comparing *A. balhimycina* Wt proteome in producing and non-producing chemostat cultivations (Table 1). This investigation revealed 20 protein spots whose abundance was positively regulated by balhimycin production in four independent differential proteome analyses. All these identified proteins - but phospho-glucosamine mutase (GlmM), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (GcpE) and a possible ATPase - are *bal* gene products (10%) or related to central carbon metabolism (40%) and cell energy and redox balance (35%) (Figure 2).

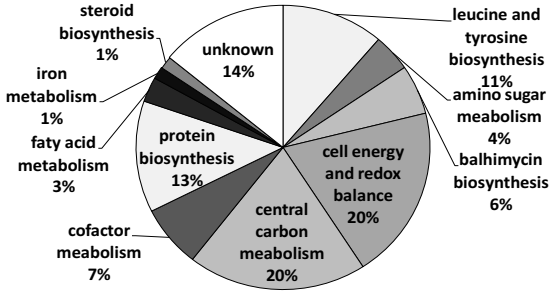

Figure 1: Relative distribution in functional classes of proteins differentially abundant both in Wt strain before and during balhimycin production and in SP1-1 and ΔoxyD *non-producing strains in the respect of Wt strain during balhimycin-producing growth-stage.*

3.2 Functional role of the balhimycin-controlled proteins

Pyruvate, acetyl-CoA and oxalacetate and 4P-erythrose are required for the synthesis of the amino acids Leu, DPG, Asn, Tyr and HPG, respectively (Figure 2). Furthermore, amino sugars necessary for the synthesis of balhimycin are synthesized from glucose. Interestingly, most balhimycin-controlled proteins are involved in glycolysis/gluconeogenesis, PP pathway and TCA cycle as the enzyme GlmM is involved in the synthesis of amino sugars. The importance of PP pathway and TCA cycle in morphological and physiological differentiation of actinomycetes, like Strepomycetes and the vancomycin-producer *Amycolaopsis orientalis*, has already been reported (Muschko et al., 2002; Ayar-Kayali and Tarhan 2010; Ayar-Kayali, 2011). Thus the activity of TCA cycle enzymes MDH, ACO and LpdA, a component of the multi enzyme pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes, could be associated with either an increased synthesis of ATP, NADPH and $FADH_2$, and a

Table 1: Relative abundances of balhimycin-regulated proteins in four independent proteome analyses

Protein (Spot N.)[a]	Gene acronym	Cellular function[b]	During Vs before production (P value)[c]	SP1-1 Vs WT (P value)[c]	Δ*oxyD* Vs WT (P value)[c]	LP Vs LG (P value)[c]
4-hydroxyphenyl pyruvate dioxygenase (129.1)	HmaS	Balhimycin biosynthesis	1.8 (P<0.0005)	0.14 (P<0.0005)	0.25 (P<0.0005)	1.5 (P<0.05)
glycosyltransferase (131.1)	BgtfB	Balhimycin biosynthesis	1.9 (P<0.0005)	0.55 (P<0.0005)	0.67 (P<0.005)	1.8 (P<0.05)
phospho-glucosamine mutase (127.1)	GlmM	Amino sugar metabolism	1.9 (P<0.0005)	0.59 (P<0.0005)	0.63 (P<0.005)	1.7 (P<0.05)
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (133.1)	GcpE	Biosynthesis of steroids	2.0 (P<0.0005)	0.59 (P<0.0005)	0.67 (P<0.005)	1.7 (P<0.01)
fructose-bisphosphate aldolase (139.1)	FDA	Glycolysis/ Gluconeogenesis	1.5 (P<0.0005)	0.67 (P<0.01)	0.67 (P<0.01)	1.5 (P<0.05)
inorganic pyrophosphatase (165.1)	PPA	Oxidative phosphorylation	1.5 (P<0.01)	0.67 (P<0.01)	0.55 (P<0.005)	1.5 (P<0.05)
NADH dehydrogenase I chain E (158.1)	NuoE	Oxidative phosphorylation	1.7 (P<0.0005)	0.38 (P<0.0005)	2.1 (P<0.0005)	1.5 (P<0.02)
thioredoxin (174.1)	TrxR	Oxidoreduction	1.5 (P<0.005)	0.5 (P<0.0005)	0.31 (P<0.005)	1.5 (P<0.03)
member of AhpC/TSA family (175.1)	AhpC	Oxidoreduction	1.7 (P<0.0005)	0.21 (P<0.0005)	0.59 (P<0.0005)	1.6 (P<0.01)
member of AhpC/TSA family (176.1)	AhpC	Oxidoreduction	1.6 (P<0.0005)	0.59 (P<0.0005)	0.67 (P<0.01)	1.6 (P<0.01)
FMN reductase luciferase like (179.1)	LUXL1	Oxidoreduction	1.8 (P<0.005)	0.67 (P<0.01)	0.43 (P<0.005)	1.5 (P<0.02)
N5,N10-methylenetetrahydromethano-pterin reductase (154.1)	LUXL2	Oxidoreduction	1.6 (P<0.005)	0.63 (P<0.005)	0.67 (P<0.005)	1.7 (P<0.005)
transaldolase (181.1)	TrA	Pentose phosphate pathway	2.4 (P<0.0005)	0.52 (P<0.005)	0.13 (P<0.0005)	1.5 (P<0.05)
transketolase (182.1)	TrK	Pentose phosphate pathway	1.6 (P<0.005)	0.29 (P<0.005)	0.67 (P<0.01)	1.5 (P<0.05)
acetyl-coenzyme A synthetase (185.1)	ACS	Pyruvate metabolism	4.8 (P<0.0005)	0.52 (P<0.0005)	0.67 (P<0.005)	2.0 (P<0.002)
dihydrolipoamide dehydrogenase (177.1)	LpdA	TCA cycle	2.8 (P<0.0005)	0.59 (P<0.005)	0.67 (P<0.005)	2.2 (P<0.005)
malate dehydrogenase (225.1)	MDH	TCA cycle	1.7 (P<0.0005)	0.67 (P<0.05)	0.65 (P<0.005)	1.7 (P<0.02)
aconitate hydratase 1 (222.1)	ACO	TCA cycle	1.7 (P<0.0005)	N. A.	N. A.	2.0 (P<0.002)
aconitate hydratase 1 (223.1)	ACO	TCA cycle	4.1 (P<0.0005)	0.67 (P<0.01)	N. d. in 42 h D*oxyD*	N. A.
possible ATPase (149.1)	ATPase	Unknown	N.d. at 18 h	0.63 (P<0.005)	0.42 (P<0.005)	1.5 (P<0.05)

[a]Numbers refers to 2D-maps reported in Gallo et al., 2010b and at the web page address http://www.unipa.it/ampuglia/Abal-proteome-maps. [b]Functional classification according to BioCyc and KEGG metabolic pathway databases. [c]Probability of null hypothesis.

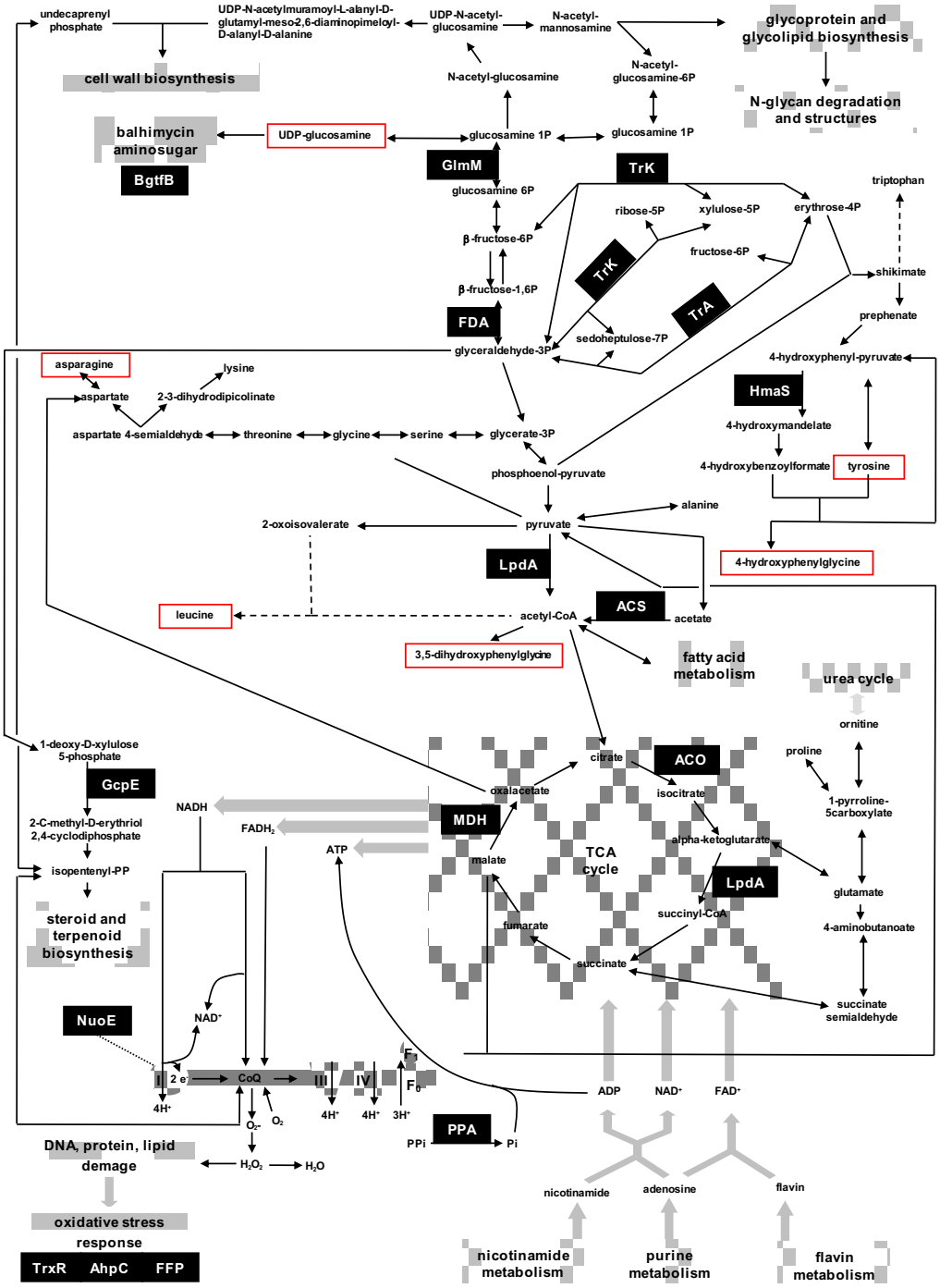

Figure 2. Scheme of metabolic pathways showing balhimycin-controlled enzymes (black boxes) Balhimycin precursors are highlighted using red rectangles. Reactions are reported according to KEGG and BioCyC metabolic pathway databases. Enzyme name abbreviations refer to Table 1.

high production of alpha-keto acids to be used in anabolic routes leading to balhimycin precursor biosynthesis (Figure 2). Also pyruvate is an important metabolic intermediate that can be converted into acetyl-CoA by LpdA or, alternatively, by acetyl-CoA synthetase (ACS). Thus acetyl-CoA can

sustain either TCA cycle and anabolic reactions (Figure 2). In addition, the PP pathway is a process that generates NADPH and pentoses. The activity of TrK and TrA enzymes, diverting metabolic intermediates towards PP pathway, leads to the synthesis of erythrose-4-P, a compound required for several biosynthetic pathways including aromatic amino acid, like Tyr, biosynthesis (Figure 2). According to the up-regulation of TCA cycle enzymes, proteins involved in energetic and redox balance processes, such as oxidative phosphorylation and oxireduction/electron transfer events, were up-regulated during antibiotic production, suggesting a correlation between energetic state and secondary metabolism production. In fact, this finding supports the hypothesis of an increased production of ATP and reduced cofactors, such as NADH and $FADH_2$, used for both biomass and antibiotic biosynthesis, coupled with an increment of proteins counteracting the damages due to reactive oxidative specie generation. In addition, the positive correlation between balhimycin production and expression of enzymes involved in redox balance,could be also related to the activation of general answer to cell stress caused by antibiotic activity. In accordance, in vancomycin resistant pathogens an increased synthesis of bacterial cell-wall is revealed and this correlates with the activity of GcpE which is required for synthesis of isopentenyl-PP. This compound is a precursor of both CoQ, involved in electron transfer, and undecaprenyl-P, involved in cell wall biosynthesis (Figure 2).

3.3 Concluding remarks

This comparative study reveals for the first time a set of proteins whose expression is positively regulated by balhimycin production in four different experimental settings. These proteins could play key roles linking central metabolism intermediates, energetic state and synthesis of antibiotic building blocks. Therefore the corresponding genes could be targeted for the construction of *A. balhimycina* synthetic strain(s) with increased antibiotic yielding.

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