RESEARCH LETTER

Novel *Amycolatopsis balhimycina* biochemical abilities unveiled by proteomics

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Abstract

Amycolatopsis balhimycina DSM5908 is an actinomycete producer of balhimycin, an analogue of vancomycin, the antibiotic of 'last resort' against multidrug-resistant Gram-positive pathogens. Most knowledge on glycopeptide biosynthetic pathways comes from studies on *A. balhimycina* as this strain, among glycopeptide producers, is genetically more amenable. The recent availability of its genome sequence allowed to perform differential proteomic analyses elucidating key metabolic pathways leading to antibiotic production in different growth conditions. To implement proteomic data on *A. balhimycina* derived from 2-DE approaches and to identify novel components, a combined approach based on protein extraction with different detergents, SDS-PAGE resolution of intact proteins and nanoLC-ESI-LIT-MS/MS analysis of their tryptic digests was carried out. With this procedure, 206 additional new proteins such as very basic, hydrophobic or large species were identified. This analysis revealed either components whose expression was previously only inferred by growth conditions, that is, those involved in glutamate metabolism or in resistance, or proteins that allow the strain to metabolize alkanes. These findings will give additional insight into metabolic pathways that could really contribute to *A. balhimycina* growth and antibiotic production and metabolic enzymes that could be manipulated to generate a model producing strain to use for synthetic biology.

Introduction

Glycopeptide antibiotics have found a successful use as last-resort antibiotics in the treatment of methicillin-resistant *Staphylococcus aureus* infections (Köck *et al.*, 2010). Leading glycopeptide drugs are vancomycin and teicoplanin, produced by the actinomycetes *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus*, respectively. In the last 15 years, *Amycolatopsis balhimycina* DSM5908, which produces the vancomycin-like antibiotic balhimycin (Pelzer *et al.*, 1999), has been investigated as model strain for studies on glycopeptide biosynthesis. Balhimycin is very similar in structure and activity to vancomycin, it shows higher activity towards anaerobic bacteria (Nadkarni *et al.*, 1994), and it has been consequently proposed as a target for future glycopeptide antibiotic production.

Balhimycin consists of a heptapeptide core made of the amino acids leucine and asparagine and the nonproteinogenic amino acids 3,5-dihydroxyphenylglycine, 4-hydroxyphenylglycine and β-hydroxytyrosine (H-Tyr). The heptapeptide is assembled by a nonribosomal peptide synthetase, and it is extensively modified by additional 'tailoring' reactions (Stegmann *et al.*, 2010). The *bal* gene cluster codes for 30 enzymes participating in the synthesis of the nonproteinogenic amino acid precursors, in the assembly of the heptapeptide scaffold and in tailoring reactions, such as the glycosylation and the cross-linking reactions of the aromatic amino acid side chains. The cross-links make the peptide scaffold rigid, creating the binding pocket for the drug target, the terminal D-Ala-D-Ala moiety of peptidoglycan (Hubbard & Walsh, 2003). In respect to other glycopeptide producers, *A. balhimycina* is more amenable to genetic manipulation, and

novel optimized derivatives of the complex molecule were generated by genetic engineering (Wohlleben *et al.*, 2009). In addition, *A. balhimycina* genome was recently sequenced (Vongsangnak *et al.*, 2012) and consists of a circular chromosome of 10.56 Mb with an average GC content of 69.9% and 8585 predicted open reading frames (ORFs).

The availability of *A. balhimycina* genome sequence and the development of high-throughput technologies, such as proteomics, allowed us to perform functional genomic studies aimed at shedding light on molecular mechanisms controlling the physiological differentiation of the strain. Therefore, differential proteomic analyses were carried out with different *A. balhimycina* strains and under various growth conditions. In particular, comparative proteomics analyses were carried out on: (1) wild-type strain before and during balhimycin production (Gallo *et al.*, 2010a); (2) two nonproducing strains, namely *A. balhimycina* SP1-1 and Δ*oxyD* (Pelzer *et al.*, 1999; Puk *et al.*, 2004; Gallo *et al.*, 2010a); and (3) wt strain grown in a chemostat in producing and nonproducing conditions (Gallo *et al.*, 2010b). These investigations showed that antibiotic production is always associated with the upregulation of either specific enzymes of balhimycin biosynthetic gene cluster or enzymes related to central carbon metabolism, cell energy and redox balance in both batch cultivations and chemostat fermentations (Gallo *et al.*, 2012a, b). In this study, a combined proteomic approach based on SDS-PAGE resolution of intact proteins and nanoLC-ESI-LIT-MS/MS analysis of their tryptic digests was carried out on protein extracts obtained using two detergents (SDS and Triton-X) to reveal whether the coding capacity of the strain was underestimated in previous 2-DE-based proteomics analysis, thus providing the molecular basis to expand our knowledge on *A. balhimycina* physiology.

Materials and methods

Bacterial strain and culture conditions

Amycolatopsis balhimycina DSM5908 strain was grown in MG medium in an orbital shaker (200 r.p.m.), at 30 °C, for 48 h, as reported in Gallo *et al.* (2010a, b). Two biological replicate MG cultures were prepared. For protein analysis, 30 mL aliquots were collected at 18 and 42 h of growth. After centrifugation, the biomasses were immediately frozen at −80 °C. For dry weight measurements of biomass, pellets from three 1-mL samples collected for each time point were dried (24 h at 65 °C) and then weighed. Glutamate and maltose were measured using the glutamine/glutamate determination kit (Sigma-Aldrich) and by following the protocol described by Hope & Dean (1974), respectively. Balhimycin production was determined with bioassays using cell-free supernatant from all the samples collected during the growth, with *Micrococcus luteus* as test organism. A calibration curve, constructed by plotting inhibition growth halos against known amounts of pure balhimycin in bioassay, was used to deduce antibiotic yield in cell-free spent medium. Specificity of the antibacterial activity in parallel bioassays was demonstrated using the specific competitor D-Ala-D-Ala added to the spent medium samples. To assess growth of *A. balhimycina* on alkanes, the strain was grown on plates containing minimal medium (Kieser *et al.*, 2000) in which glucose was replaced by n-hexadecane or n-dodecane (2.26 g L^{-1}) as sole carbon sources.

Protein separation and digestion

Thirty milliliter aliquots of two replicated cultivations were collected at 18 and 42 h of growth. After centrifugation, the biomasses were immediately frozen at −80 °C. Frozen biomass samples were sonicated using a 3% SDS- or a 2% Triton-X-containing buffer, as already described (Gallo *et al.*, 2010a). Two protein pools were made by combining equal amount of proteins from each replica taken at 18 and 42 h of growth and then analysed by 12% SDS-PAGE (14 cm × 16 cm × 0.75 mm). Electrophoresis was performed in an SE600 vertical electrophoresis system (Hoefer), at 18 °C, using a constant current setting of 25 mA and a maximum of 150 V. All electrophoretic reagents were from Bio-Rad. After electrophoretic separation, proteins were visualized by silver nitrate staining (Shevchenko *et al.*, 1996). Digitalized gel images were acquired using an Image Scanner III (GE Healthcare) apparatus and analysed by the IMAGE MASTER 2D Platinum 6.0 software (GE Healthcare), according to the manufacturer's instructions. Independent lanes from SDS-PAGE were cut separately and each subdivided into 18 similar gel portions, which were then independently triturated, washed with water, in-gel reduced, S-alkylated, and digested with trypsin (Sigma, sequencing grade). Resulting peptide mixtures were desalted by μZip-TipC18 (Millipore) using 50% (v/v) acetonitrile, 5% (v/v) formic acid as eluent (Buanne *et al.*, 2013) and directly analysed by nanoLC-ESI-LIT-MS/MS.

Mass spectrometry (MS) and bioinformatic analysis

Peptide mixtures from 1-DE slices were analysed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (ThermoFisher, San Jose, CA) equipped with a Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Odense, Denmark) (Gallo *et al.*, 2010b). Peptide mixtures were separated on an Easy C18 column

(10 × 0.075 mm, 3 μm) (Proxeon). Mobile phases were 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B), running at a total flow rate of 300 nL min^{-1}. Linear gradient was initiated 20 min after sample loading; solvent B ramped from 5% to 35% over 45 min, from 35% to 60% over 10 min and from 60% to 95% over 20 min. Spectra were acquired in the range *m*/*z* 400–2000. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 60 s); the mass isolation window and collision energy were set to *m*/*z* 3% and 35%, respectively.

Raw data files from nanoLC-ESI-LIT-MS/MS experiments were searched by MASCOT search engine (version 2.2, Matrix Science, UK) to identify proteins from gel slices, by comparison of tryptic peptide product ion mass spectra against those generated from an NCBI nonredundant database also containing the *A. balhimycina* ORF product database based on *A. balhimycina* DSM5908 genome sequencing (*A. balhimycina* GenBank entry acc. no.: NZ_KB913037). Database searching was performed using Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively, a mass tolerance value of 2.0 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme and a missed cleavage maximum value of 2. Other MASCOT parameters were kept as default. Candidates with at least 2 assigned peptides with an individual confidence level of 95% were considered as properly identified. Definitive peptide assignment was always associated with manual spectra visualization and verification.

Results and discussion

Experimental setup

When incubated in MG liquid medium, *A. balhimycina* showed a reproducible biphasic growth kinetics (Fig. 1a), with a first rapid growth phase (RG1, 16–22 h), a transition phase (T, 22–40 h), a second rapid growth phase (RG2, 40–46 h) and a stationary phase (after 48 h). Microbiological assays revealed that balhimycin production started at 22 h, its titre gradually increased until 42 h, with a final concentration of about 25 mg L^{-1} (Fig. 1a). During growth, glutamate was consumed as primary carbon and nitrogen source causing a pH increment (from 6.5 to 7.3 at 48 h), while only modest amounts or no maltose was consumed (Fig. 1b). Proteins were extracted with a SDS-containing or a Triton-X-containing buffer from biomass samples collected before (18 h) and during balhimycin production (42 h); to make the reading easier, the protein samples were defined as 18-SDS, 42-SDS, 18-Tx and 42-Tx, corresponding to proteins extracted at 18 and 42 h, using a SDS-containing or a Triton-X-containing extraction buffer, respectively. All samples were loaded on 12% SDS-PAGE for protein separation (data not shown). Eighteen polyacrylamide gel slices per sample were generated and subjected to tryptic digestion for tandem MS procedures. Although not quantitative, this approach allowed to recognize most abundant proteins in each sample, thus providing information on induced or repressed components under a specific growth condition. In addition, the detergents used in this study facilitated the identification of proteins with various physico-chemical properties, which escaped previous proteomic analyses of *A. balhimycina* for their high pI value, high molecular mass value, high hydrophobicity value or other technological reasons (Gallo *et al.*, 2010a, b, 2012b).

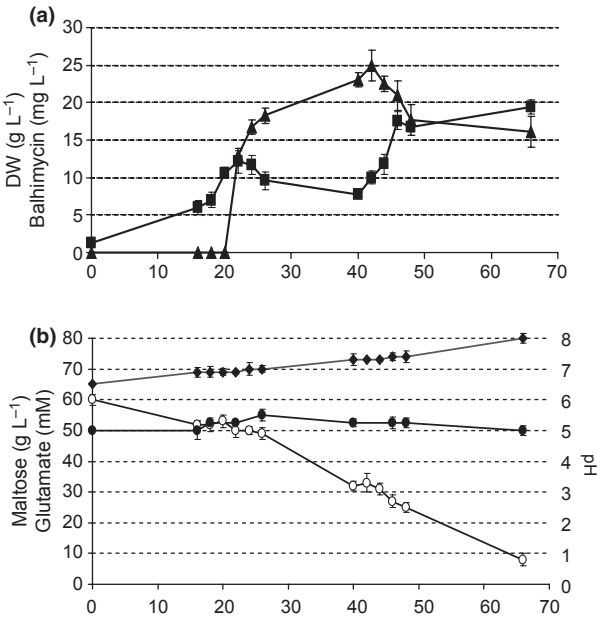

Fig. 1. (a) Growth (■) and balhimycin (▲) production of *Amycolatopsis balhimycina*. (b) pH values (♦), glutamate (○) and maltose (●) consumption during growth.

In particular, MS analysis identified 331 proteins in 18-SDS, 313 in 42-SDS, 223 in 18-Tx and 300 in 42-Tx. Considering the redundancy of some protein identifications between gel bands, MS analysis revealed a total of 115, 148, 115 and 143 proteins in 18-SDS, 42-SDS, 18-Tx and 42-Tx, respectively (Fig. 2a). As 66 proteins of the 235 extracted with SDS (Supporting Information, Table S1) and 57 of 225 extracted with Triton-X (Table S2) were previously identified by 2-DE-based proteomics (Gallo *et al.*, 2010a, b), this study led to the identification of novel 206 proteins (Fig. 2b). By removing intrinsic limitations due to 2-DE (Salzano *et al.*, 2007), the proteomic approach used here allowed to identify 53 basic proteins (with a predicted

pI larger than 10), 11 large proteins (with a predicted molecular weight larger than 100 KDa) and hydrophobic proteins. In the case of basic proteins, for example, this is due to the fact that commercial strips do not overcome a pH value of 11. On the other hand, large/hydrophobic proteins often do not easily enter the polyacrylamide network of the 2-DE gels, limiting their presence therein. Conversely, as most of the actinomycetes proteins focus in the specific range of pI and mass values (specifically pI 4–6 and Mr 30–60 kDa, respectively), the approach used in this study did not solve the problem of identifying poorly abundant proteins migrating in these arrays that, although with a crowded distribution, were better resolved (and identified) under 2-DE. Proteins were further clustered into functional groups, according to BIOCYC (http://www.biocyc.org/), KEGG (http://www.genome.ad.jp/kegg/kegg2.html) and EXPASY (http://www.expasy.org) metabolic pathway databases. Here, we discuss the newly identified proteins. On the basis of their expression profile, proteins were classified in three groups: A, B and C.

Group A proteins

Group A (Table S3) contains 101 identified in both time growth phases (18 and 42 h). Among these, 43 proteins are involved into protein biosynthesis, folding and modification, for example 38 ribosomal proteins, translational initiation factors IF2 and IF3, and the molecular chaperone HtpG. Eight proteins are related to amino acid biosynthesis and membrane transport (i.e. glutamate ABC transporter glutamate-binding protein, glutamate transport system ATP-binding protein, glutamate dehydrogenase). Their expression was previously only inferred on the basis of the microorganism metabolism. Indeed, notwithstanding the essential role of glutamate as primary source of carbon and nitrogen for the growth of *A. balhimycina* (Fig. 1a), previous proteomic characterizations did not reveal any proteins devoted to glutamate metabolism (Gallo *et al.*, 2010a). In contrast, this study identified a NAD-specific glutamate dehydrogenase, a key enzyme to grow on glutamate as sole carbon source. This enzyme catalyses the conversion of glutamate into alpha-ketoglutarate and NH^{3+} and was missed from previous 2-DE-based proteomic investigations because of its molecular weight (175–180 kDa). Besides glutamate dehydrogenase, glutamate ABC transporter glutamate-binding protein and glutamate transport system ATP-binding protein were also identified. Having a theoretical pI value of about 9.7, the first one was not revealed before, while the second one focused in a crowded region of the 2-DE gel (pI *c.* 7 and mass 27 kDa).

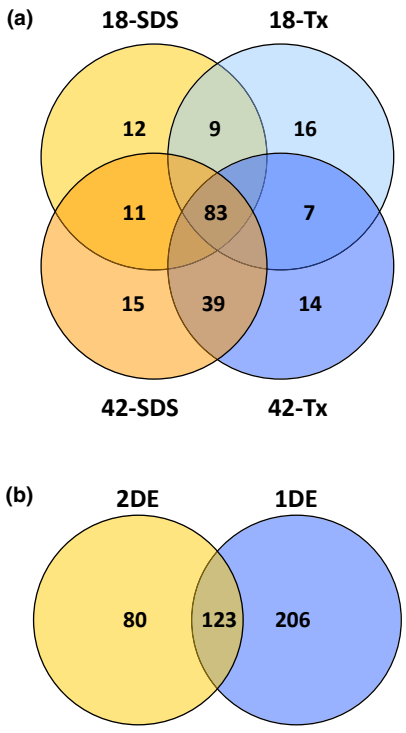

Fig. 2. Summary of proteins detected by 1DE-proteomics in 18-SDS, 42-SDS, 18-Tx and 42-Tx samples (a) and comparision of the total number of proteins identified by 1DE- and 2DE-proteomics (b).

Eleven proteins are enzymes involved in carbohydrate metabolism (fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, dihydrolipoamide dehydrogenase, glucose-6-phosphate isomerase, D-xylose transport system substrate-binding protein, citrate synthase, 2-oxoglutarate dehydrogenase, dihydrolipoamide succinyltransferase, fumarate hydratase, succinate dehydrogenase, glycerol kinase). Six proteins are devoted to metabolism of nucleotide, cofactors and vitamins, such as 4-hydroxybenzoate polyprenyltransferase-like prenyltransferase (involved in ubiquinone biosynthesis), molybdenum cofactor synthesis domain protein, nucleotide-binding protein, inositol 1-phosphate synthase, uroporphyrinogen-III synthase/uroporphyrinogen-III C-methyltransferase, guanosine pentaphosphate synthetase I/polynucleotide phosphorylase. Seven proteins correspond to enzymes involved in oxidative phosphorylation, such as subunits of ATP synthase F0 and F1 and NADH dehydrogenase, while one is thioredoxin. Sixteen proteins are devoted to other processes, such as transcription or transcriptional regulation, that is HU-Beta, transcription termination factor Rho, the two subunits beta and beta' of DNA-directed RNA polymerase and polynucleotide phosphorylase (PNPase). Their identification would not have been possible by 2-DE-based proteomics as they are very basic or have a high mass value. In particular, HU-beta is a histone-like DNA-binding protein that is capable of wrapping DNA to stabilize it, thus preventing its denaturation under extreme environmental conditions. On the other hand, PNPase is

an enzyme that catalyses the 3′–5′ phosphorolysis of RNAs and can also produce ribopolymers, using nucleoside diphosphates as substrates. In actinomycetes, together with RNaseE, the primary endoribonuclease involved in degradation/processing of mRNAs and stable RNAs, PNPase is the major component of the degradosome, the multiprotein complex involved in RNA turnover (Bandyra *et al.*, 2013). In this context, the most studied example comes from *Nonomuraea* sp. ATCC 39727, the producer of the glycopeptide antibiotic A40926, where PNPase plays a major role in RNA degradation, by controlling RNA half-life of biosynthetic genes and, as a consequence, the antibiotic production (Siculella *et al.*, 2010). Many ABC transporters and integral membrane proteins have been found; their role is difficult to be addressed and it could be investigated in well-known hosts, such as *Streptomyces lividans* (Giardina *et al.*, 2010), where actinomycete genes can be easily transferred and expressed (Alduina *et al.*, 2003, 2005).

An aliphatic sulphonates family ABC transporter and a protein involved in aminosugar metabolism, namely galactofuranosylgalactofuranosylrhamnosyl-N-acetylglucos aminyl-diphospho-decaprenol beta-1,5/1,6-galactofuranosyltransferase, were also identified. The latter was demonstrated to be important for the assembly of mycobacterial arabinogalactan (Wheatley *et al.*, 2012).

Finally, nine of the 101 are proteins with unknown function.

Group B proteins

Group B (Table S4) includes 37 new proteins detected before antibiotic production (18 h). Five proteins are related to amino acid metabolism and transport (aspartate aminotransferase, O-acetylhomoserine (thiol)-lyase, amino acid transporter, diaminobutyrate acetyltransferase and serine hydroxymethyltransferase); in particular, they are devoted to metabolism/transport of aspartate, cysteine, glycine, serine and threonine. While a protein is involved in fatty acid biosynthesis (3-oxoacyl-ACP reductase), three are related to metabolism of nucleotide, cofactors and vitamins (RNaseJ, ribonuclease PH and a nucleoside-binding protein), three in oxidative phosphorylation (flavin-dependent oxidoreductase, cytochrome C oxidase and NADH-quinone oxidoreductase subunit L), four in oxidoreduction processes (short-chain dehydrogenase/reductase SDR, aerobic-type carbon monoxide dehydrogenase, short-chain dehydrogenase of unknown substrate specificity and short-chain alcohol dehydrogenase-like protein) and eight in protein biosynthesis, folding and modification (peptidyl-prolyl cis-trans isomerase, ATP-dependent Clp protease ATP-binding subunit ClpC, glycyl-tRNA synthetase, the small subunit ribosomal protein S10, preprotein translocase subunit SecD, valyl-tRNA synthetase, arginyl-tRNA synthetase and 50S ribosomal protein L25). Finally, six proteins are devoted to carbohydrate metabolism (succinate dehydrogenase hydrophobic anchor subunit-like protein, malate dehydrogenase, beta-mannosidase, trehalose 6-phosphatase, glutamine-fructose-6-phosphate transaminase, glucose-6-phosphate 1-dehydrogenase), while an ABC-type transport system for nitrate/sulphonate/bicarbonate, an ABC transporter for aliphatic sulphonates and five proteins with unknown function were also identified.

Group C proteins

Group C (Table S5) comprises 68 proteins that were only detected during antibiotic production (42 h). Four proteins are related to amino acid metabolism (tetrahydrodipicolinate N-succinyltransferase, aspartyl-tRNA synthetase, leucyl aminopeptidase, carbamoyl-phosphate synthase L chain ATP-binding protein). Seven proteins are involved in carbohydrate metabolism (multiple sugar transport system substrate-binding protein, pyruvate dehydrogenase E1 component, malate dehydrogenase, UDP-galactopyranose mutase, UDP-glucose pyrophosphorylase, aldo/keto-reductase), while three-ones are related to fatty acid biosynthesis (acyl-ACP desaturase, enoyl-acyl carrier protein reductase and ACP of an fatty acid synthase) and one to lipid metabolism (phospholipid-binding protein). Twelve proteins are related to other processes, such as cell division and DNA replication, transcriptional regulation, DNA damage and alkane metabolism; seven-ones are related to the metabolism of nucleotides, cofactors and vitamins, such as molybdopterin, pyridoxal and thiamine S, molybdenum cofactor synthesis domain protein and thioredoxin-disulphide reductase. Two proteins are involved in oxidative phosphorylation (ATP synthase F1 subcomplex epsilon subunit and cytochrome b subunit); eighteen proteins are devoted to protein biosynthesis, folding and modification (ribosomal proteins L19, L31, S12, S15, S18 and S30, protein translocase subunit SecF, cold-shock DNA-binding protein family, cold-shock protein, bacterial peptide chain release factor 2, prolyl-tRNA synthetase, family II, leucyl-tRNA synthetase and isoleucyl-tRNA synthetase). Twelve proteins with unknown function have been also identified.

Interestingly, two proteins related to secondary metabolism were also detected at 42 h, namely VanA D-alanine-D-lactate ligase and a polyketide cyclase/dehydrase. Polyketides (macrolides, tetracyclines and polyenes) are secondary metabolites present in actinomycetes, which are generally biosynthesized through the decarboxylative condensation of malonyl-CoA-derived extender units similar to fatty acid synthesis. Polyketide chains produced by the

minimal polyketide synthase are further derivatized and modified into bioactive natural products. Genome sequence revealed that actinomycetes contain about 20–25 silent biosynthetic gene clusters for either polyketides or nonribosomal peptides. On the other hand, VanA, encoded into the operon *vanHAX*, is the D-alanine-D-lactate ligase responsible for the production of a cell wall containing the D-Lac-ending precursor, which makes the microorganism resistant to its own glycopeptide product (Schäberle *et al.*, 2011). Interestingly, our proteomic approach led to the identification of an alkanesulphonate monooxygenase putatively required for the degradation of aliphatic compounds. In addition, expression of an ABC transporter for aliphatic sulphonates strongly suggested that *A. balhimycina* may grow on alkanes. To address this point, the strain was grown on minimal media containing n-hexadecane and n-dodecane as sole carbon sources (data not shown). The strain grew well demonstrating that the information derived from a simple proteomic observation can be very useful to get information on new biochemical abilities and the lifestyle of *A. balhimycina*. This activity is not present in the model actinomycete *S. coelicolor,* which is able to use n-hexadecane and n-dodecane as sole carbon sources only after its previous transformation with the alkane monooxygenase-coding gene (Gallo *et al.*, 2012a).

In conclusion, in this work, we used a proteomic approach based on biomass extraction with two detergents (SDS and Triton-X), followed by SDS-PAGE resolution of intact proteins and nanoLC-ESI-LIT-MS/MS analysis of their tryptic digests to implement the proteomic data available on *A. balhimycina*, a microorganism that is more amenable to genetic strain improvement with respect to other glycopeptide-producing actinomycetes (Alduina *et al.*, 2005, 2007; Alduina & Gallo, 2012). A deeper knowledge about *A. balhimycina* biochemical pathways could be used to generate model strains with new activities and new functions by synthetic biology (Alduina & Gallo, 2012; Wohlleben *et al.*, 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Proteins extracted with SDS, already found by 2DE-Proteomics.
Table S2. Proteins extracted with TritonX, already identified by 2DE-proteomics.
Table S3. Summary of Group A proteins.
Table S4. Summary of Group B proteins.
Table S5. Summary of Group C proteins.