Quantitative Proteomics Analysis of *Streptomyces coelicolor* Development Demonstrates That Onset of Secondary Metabolism Coincides with Hypha Differentiation*[S]

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Streptomyces* species produce many clinically important secondary metabolites, including antibiotics and antitumorals. They have a complex developmental cycle, including programmed cell death phenomena, that makes this bacterium a multicellular prokaryotic model. There are two differentiated mycelial stages: an early compartmentalized vegetative mycelium (first mycelium) and a multinucleated reproductive mycelium (second mycelium) arising after programmed cell death processes. In the present study, we made a detailed proteomics analysis of the distinct developmental stages of solid confluent *Streptomyces coelicolor* cultures using iTRAQ (isobaric tags for relative and absolute quantitation) labeling and LC-MS/MS. A new experimental approach was developed to obtain homogeneous samples at each developmental stage (temporal protein analysis) and also to obtain membrane and cytosolic protein fractions (spatial protein analysis). A total of 345 proteins were quantified in two biological replicates. Comparative bioinformatics analyses revealed the switch from primary to secondary metabolism between the initial compartmentalized mycelium and the multinucleated hyphae. *Molecular & Cellular Proteomics 9:1423–1436, 2010.

Streptomyces is a Gram-positive bacterium characterized by a complex development cycle. Detailed biochemical and confocal laser microscopy analyses of the *Streptomyces* developmental cycle recently performed by our group demonstrated novel aspects of the differentiation processes of this bacterium (1–7). A previously unidentified compartmentalized mycelium (MI)[1] initiates the developmental cycle and then undergoes a PCD in a highly ordered morphological and biochemical sequence of events (1, 2, 4). This PCD is a lytic process that substantially differs from eukaryotic PCD (apoptosis) (3, 4). Viable segments of the first compartmentalized hyphae begin to enlarge as a multinucleated mycelium (MII) that grows in successive waves that determine the characteristic complex growth curves of this microorganism. Two types of second mycelium were defined based on the absence (in early development) or presence (in late development) of the hydrophobic layers distinctive of aerial hyphae (5). The traditionally denominated substrate (vegetative) mycelium corresponds to the early second multinucleated mycelium (5). It has been proposed that the first compartmentalized mycelium fulfills the true vegetative role in *Streptomyces* development in soil (6). In this view, the second early and the late multinucleated mycelia could be considered as a single stage of the reproductive phase because they are destined to sporulate (6).

Streptomyces is an extremely important bacterium in biotechnology because approximately two-thirds of industrial antibiotics are synthesized by members of this genus (8). Streptomycetes also produce large numbers of eukaryotic cell differentiation regulators, including apoptosis inhibitors and inducers (9–11). Some researchers hypothesize that bacteria with complex life cycles (streptomycetes, cyanobacteria, etc.) represent the evolutionary origin of some of the protein domains involved in PCD processes, including eukaryotic apoptosis: apoptotic (AP) ATPases, cysteine-aspartic acid proteases (caspases and effector and regulatory apoptotic

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[1] The abbreviations used are: MI, compartmentalized mycelium; MII, multinucleated mycelium; PCD, programmed cell death; AP, apoptotic; PLGS, ProteinLynx Global server; iTRAQ, isobaric tags for relative and absolute quantitation; TIR, Toll/IL-1 receptor; UPLC, ultraperformance LC; CV, coefficient of variation; KEGG, Kyoto Encyclopedia of Genes and Genomes; NCBInr, National Center for Biotechnology Information non-redundant; ABC, ATP-binding cassette; ARC, AAA ATPase forming ring-shaped complexes; CAP, catabolite activator protein.

proteases), Toll/IL-1 receptor (TIR) domains (eukaryotic PCD adaptor molecules), Ser/Thr kinases, etc. (12–17). *In silico* analysis of the *Streptomyces coelicolor* genome predicted the occurrence of 40 eukaryotic-type Ser/Thr kinases (16), one caspase (15), and several AP ATPases and proteins harboring TIR domains (15); *S. coelicolor* is one of the bacteria with the greatest numbers of these types of eukaryotic-like molecules. The biological function of most of these proteins remains unknown in bacteria.

Streptomyces biology has been studied using proteomics approaches in various cellular contexts, including programmed cell death (8), germination (17, 18), mutant analysis (bald A mutant) (19–21), phosphate limitation (22), and the diauxic lag phase (23). Most of these experiments only gave qualitative information, using mainly two-dimensional gel electrophoresis for protein separation followed by mass spectrometry for protein identification. Recently, two independent transcriptomics analyses about *Streptomyces* differentiation in submerged cultures were performed (24, 25), and in one of them a quantitative proteomics study using stable isotope labeling and LC-MS/MS was performed in combination with transcriptomics to analyze genes and proteins with divergent mRNA-protein dynamics in submerged cultures of this bacterium (24).

In the present work, we performed a detailed quantitative proteomics analysis of *S. coelicolor* A3(2) solid cultures in which *Streptomyces* carried out a complete developmental cycle (5, 26). Using iTRAQ and LC-MS/MS, we aimed to reveal differences in the *Streptomyces* proteome along the developmental mycelial stages, including also the study of membrane and cytosolic protein fractions. In the course of this process, we created a database of protein expression profiles during the *Streptomyces* developmental phases that will facilitate further analysis of the regulation of these complex events.

EXPERIMENTAL PROCEDURES

Strain and Medium—S. coelicolor M145 was used in this study. Petri dishes (8.5 cm) with 25 ml of solid glucose, yeast/malt extract medium (4) were covered with cellophane disks, inoculated with 100 μl of a spore suspension (1×10^7 viable spores/ml), and incubated at 30 °C. This medium promotes the rapid development of a lawn that readily differentiates and yields abundant sporulation.

Sampling and Fractionation of S. coelicolor Cells throughout Differentiation Cycle—Mycelium was scraped off from plates at different time points (12, 24, and 72 h) using a plain spatula (see Figs. 1 and 2). Two independent series of cultures were prepared and processed (biological replicates) with the same batch of spores and medium and developed simultaneously in the same incubator. At 12 h, the first compartmentalized mycelium was separated from the non-septate mycelium by conversion of the cell compartments to protoplast forms as described previously (3). Cell-free extracts were obtained by osmotic shock in buffer A (50 mM Tris-HCl, pH 7, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, and 0.5 mM PMSF) and sonication on ice. Samples of second mycelium were obtained at the phases in which the first compartmentalized mycelium has died (24 and 72 h) (1, 2). Mycelial pellets were mechanically disaggregated (strong vortexing for 1 min) in buffer A (2.5 g of mycelium in 10 ml; 12 Petri dishes at 24 h and 10 plates at 72 h) precooled at 0 °C and centrifuged for 10 min at 5000 $\times$ *g* at 4 °C. Mechanical disaggregation and washing steps were repeated eight times. Cells were broken up in an MSE Soniprep 150 in four cycles of 10 s on ice. The unbroken cells and cellular debris were eliminated by centrifugation (7740 $\times$ *g*) at 4 °C for 15 min.

Subcellular fractionation was performed simultaneously in the two biological replicates using the same reagents and under the same experimental conditions. Cytosolic and membrane fractions were obtained according to Quirós *et al.* (27) by ultracentrifugation at 100,000 $\times$ *g* in a Beckman LB-70 M ultracentrifuge. Membranes were resuspended in buffer A and incubated at 0 °C for 30 min with periodical vortex shaking. Membranes were subsequently ultracentrifuged again at 100,000 $\times$ *g*. This process was repeated three times, discarding the supernatants. Membranes were later resuspended in 100 mM Na_2CO_3 (pH 11) and washed two more times. These three supernatants were collected and corresponded to the extrinsic membrane proteins. Finally, the membranes were washed two times with buffer A without salt. These membranous pellets corresponded to the intrinsic membrane proteins that were not delipidated. Supernatants of the washing steps in Na_2CO_3 (extrinsic membrane proteins) were collected and dialyzed (Sigma D7884 benzoylated cellulose tubing) against buffer A at 4 °C for 1 h with four buffer changes. Membrane fractions were stored at −80 °C.

Viability Staining—The permeability assay described previously for *Streptomyces* was used to stain the cultures (12). The samples were observed under a Leica TCS-SP2-AOBS confocal laser-scanning microscope at a wavelength of 488- and 568-nm excitation and 530- (green) or 640-nm (red) emissions.

Protein Separation, Digestion, and iTRAQ Labeling of Peptides—Protein quantification was performed using the Bradford (28) and Lowry (29) assays using bovine serum albumin as a standard (Sigma). Proteins, 50 μg/lane, were separated by SDS-PAGE using precast PAGEr® 4–20% Tris-glycine gels (Lonza) and stained with Coomassie Blue (Coomassie Brilliant Blue G-250). When necessary, samples were concentrated by filtration using Vivaspin 20 (10,000 molecular weight cutoff, Sartorius). For intrinsic membrane proteins, membranes containing 50 μg of protein were boiled in the SDS loading buffer for 5 min and run directly in the gel. The three samples (MI/12h, MII/24h, and MII/72h) of each subcellular fraction and the two biological replicates were loaded in six different gels, which were used for six independent iTRAQ triplex experiments: cytosolic, membrane intrinsic, and membrane extrinsic proteins from two biological replicates. Each gel lane was divided into six slices with a scalpel. Gel slices were cut into small pieces, washed with distilled water, and shrunk with acetonitrile. Cys residues were reduced with DTT and *S*-alkylated with iodoacetamide; swelled with a solution of 10 ng/μl trypsin (Promega), 50 mM triethylammonium bicarbonate digestion buffer; and incubated overnight at 37 °C. After digestion, supernatants were recovered, and remaining peptide extractions from gel fragments were performed with a volume of 5% formic acid for 30 min after which an equal volume of pure acetonitrile was added, and the samples were incubated for an additional 30 min at room temperature. Extracts were vacuum-dried. Peptides were labeled with iTRAQ eightplex reagent (Applied Biosystems, Foster City, CA) according to our previously reported protocol (30): 113, 114, and 115 iTRAQ tags were used for the 12-, 24-, and 72-h samples, respectively. After labeling for 2 h at room temperature, samples were combined (six samples corresponding to the original gel pieces). The concentration of organic solvent was reduced using a vacuum concentrator, and peptide desalting was performed using GELoader micropipette tips (Eppendorf) prepared with C_{18} (Empore extraction disks, 3M) and R3 material (see Fig. 2).

Analysis of iTRAQ-labeled Peptides by Nano-LC-Tandem Mass Spectrometry—Tryptic peptides were separated using a NanoAcquity UPLC system (Waters) modified with a 2.6-μl PEEKsil sample loop (SGE Analytical Science, Darmstadt, Germany). Mobile phase A was 0.1% formic acid in double distilled H_2O, and mobile phase B was 0.1% formic acid in 90% acetonitrile (Fisher Scientific). A 2.5-μl sample was injected and loaded into the Bridged ethyl hybrid C_{18} column (1.7 μm, 15-cm $\times$ 75-μm analytical reversed phase column, Waters) in direct injection mode with 3% B for 10 min at 400 nl/min. Peptides were eluted from the column with a linear gradient of 3–7% B for 4 min, 7–30% B for 60 min, 30–60% B for 15 min, and 60–90% for 5 min at a flow rate of 300 nl/min. The column was washed with 90% B for 10 min followed by equilibration for 14 min at a flow rate of 400 nl/min. The column temperature was kept at 36 °C. The lock mass solution for MS and MS/MS comprised 500 fmol/μl [Glu^1]fibrinopeptide B (Sigma) and was delivered by the auxiliary pump of the NanoAcquity at a constant flow rate of 500 nl/min to the reference sprayer of the NanoLockSpray source of the mass spectrometer.

The UPLC system was interfaced to a Q-TOF tandem mass spectrometer (SYNAPT, Waters). The mass spectrometer was operated in positive ion mode at a mass resolution of ~10,000 full width at half-maximum. The TOF analyzer (v-mode) of the mass spectrometer was externally calibrated with [Glu^1]fibrinopeptide B fragment ions from *m/z* 50 to 1500. Acquired data were postcalibrated using the doubly protonated precursor ion of [Glu^1]fibrinopeptide B. The reference sprayer was sampled every 120 s. LC-MS/MS data were obtained using a data-dependent acquisition method. MS survey analysis was performed for 0.48 s with an interscan delay of 0.02 s followed by two MS/MS cycles. The fragment ions from the two most abundant multiply charged precursor ions (2+, 3+, and 4+) were detected at an integration rate of 0.48 s with a 0.02-s interscan delay. The collision energy was ramped from 20 to 45 eV. The dynamic exclusion of precursors was set to 60 s. Each sample was analyzed twice; the second LC-MS/MS analysis was performed with the exclusion list from the precursor *m/z* values selected for the first LC-MS/MS analysis.

LC-MS/MS Data Analysis—ProteinLynx Global server (PLGS) program version 2.3 was used to convert LC-MS/MS raw data into pkl files. pkl files were submitted for search by the MASCOT search engine (version 2.2) against the NCBInr database with taxonomy limited to *S. coelicolor* (January 22, 2009, 8537 entries). The following MASCOT search parameters were used: peptide mass tolerance, 10 ppm; fragment mass tolerance, 0.1 Da; trypsin cleavage with a maximum of two missed cleavages; fixed modifications, *S*-carbamidomethyl on cysteine and iTRAQ on lysine residues and N termini of peptides; variable modification, oxidation on methionine. Example spectra are shown in supplemental Fig. 1. Peptide false positive rates were calculated using the decoy option provided by MASCOT (with the combined pkl file) resulting in 1.33–1.36% false positives for the first biological replicate and 1.06–1.08% for the second biological replicate (identity and homology thresholds, respectively; significance threshold, 0.016).

Relative quantification was performed using PLGS (Waters) with automatic normalization. The PLGS quantification algorithm uses Bayesian Markov chain Monte Carlo methods to explore the posterior probability and takes the different scores of individual peptides from a protein into account to quantify expression changes. Results obtained from PLGS were exported into Microsoft Excel for further computational and bioinformatics data analysis. Proteins that were not represented by any peptide above the MASCOT homology threshold were discarded. When a protein was detected in more than one of the six gel slides processed, the protein with the highest MASCOT score was retained. The three samples (MI/12h, MII/24h, and MII/72h) of each subcellular fraction and each biological replicate were processed independently. Consequently, we performed six independent iTRAQ triplex experiments: cytosolic, membrane intrinsic, and membrane extrinsic proteins from two biological replicates. We also performed six independent relative quantifications using PLGS, and we estimated the relative abundance values between the three developmental stages analyzed for the proteins from the same subcellular fraction and biological replicate.

Technical variability was tested by running one sample (cytosolic fraction, 24 h) in identical portions (duplicate) in the same gel followed by labeling with two iTRAQ reagents (113 and 114 *m/z*) and LC-MS/MS analysis. Biological variability was tested by means of parallel analysis of two independent biological replicates in one gel, labeling them with the same iTRAQ reagent and analyzing them in two independent LC-MS/MS experiments. For proteins identified in both biological replicates, iTRAQ ratios were considered significant if their average in both replicates ($\pm$S.D.) were greater or lower than unity. With respect to the remaining proteins, iTRAQ ratios values were considered significant if their coefficient of variation (CV) was less than 0.25. Consequently, we kept protein abundance values with good reproducibility between biological replicates (CV $<$ 0.25) as well as those with CVs higher than 0.25 but with averaged iTRAQ ratios that varied significantly between mycelial stages (average iTRAQ ratios $\pm$ S.D. above or below unity); we discarded the remaining proteins (protein abundance values without good reproducibility between biological replicates). All identified proteins that satisfied these strict criteria were considered for further analysis (supplemental Tables 1–4) and were included in the results and figures reported in this study (see below). Spectra of peptides from proteins identified with a single peptide are shown in supplemental Fig. 3.

ProteinCenter 2.0 (Proxeon) was used to conduct the computational and bioinformatics data analyses and protein classification. Proteins were classified into functional categories according to their annotated functions in GenBank™ and by homology/functions according to the gene ontology, the conserved domain, and the KEGG pathway databases.

Cluster Analysis of Protein Expression Profiles—The averaged iTRAQ values obtained in two biological replicates for each protein at all three time points (12, 24, and 72 h) were $\log_2$-transformed. Data were normalized to obtain a mean value of 0 and a standard deviation of 1, ensuring that proteins with similar expression patterns could be easily compared without taking into account their absolute values. For clustering, we used the fuzzy *c*-means algorithm with a Euclidean distance matrix (31). This method groups the data into *c* protein clusters with the most similar patterns by minimizing an objective function. The results provide *c* membership values for each protein. A membership value gives a measure in the range (0, 1) of how strongly the expression pattern of a protein follows the one of the cluster center. We associated each protein to the cluster for which it had the largest membership value. Changes in the input parameter *m*, the so-called "fuzziness," did not give different results within the parameter range $m = 1.1–2$. We therefore defined $m = 1.2$. The optimum value for the other parameter, the number of clusters (*c*), was determined by comparing the Xie-Beni index (32) calculated from the corresponding results.

RESULTS

Fractionation of Specific Mycelial Stages in S. coelicolor—At any specific developmental time point, different mycelial stages coexist with PCD phenomena (3). We have previously reported a method by means of which dead and live cell samples are enriched on the basis of protoplast formation from the first compartmentalized mycelium under-

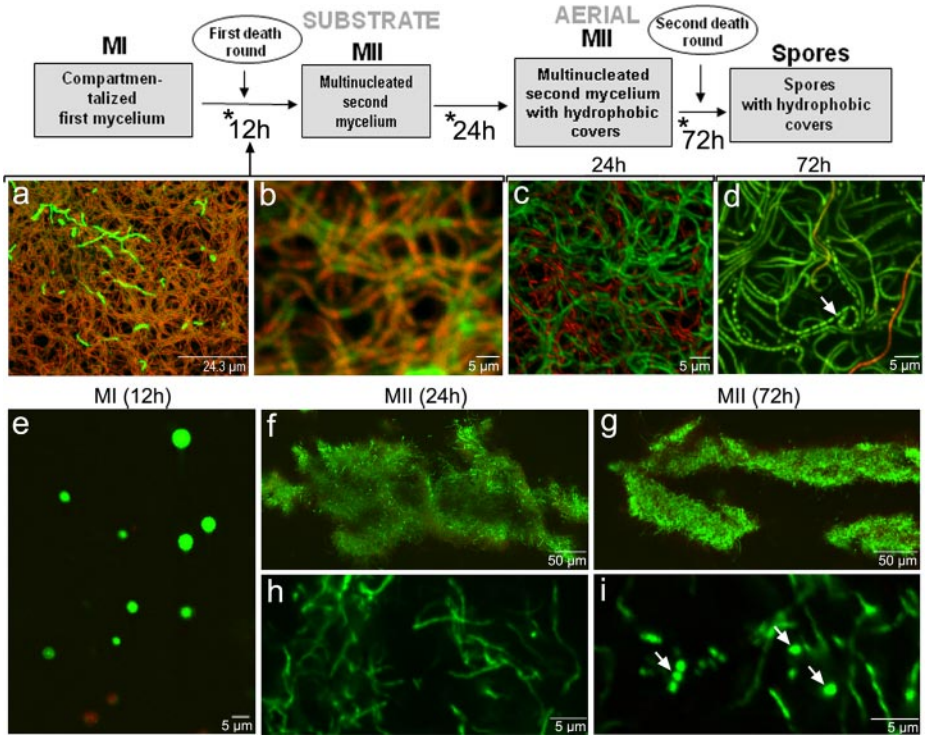


FIG. 1. **Sample preparation.** *Upper panel*, cell cycle features of *Streptomyces* development. Mycelial structures (MI, first compartmentalized mycelium; MII, second early and late multinucleated mycelia), cell death processes, and traditional denomination ("substrate" and "aerial") are indicated. Developmental time points at which samples were collected are indicated by an *asterisk*. *a–d*, confocal laser-scanning fluorescence microscopy analysis of horizontal sections of cultures at the phases collected for proteomics analyses. Developmental time points are indicated. Samples were stained with SYTO 9 and propidium iodide (see "Experimental Procedures"). *e–i*, confocal laser fluorescence micrographs of the different mycelia after processing (see "Experimental Procedures"). Mycelial types and developmental time points are indicated. See text for details.

going early PCD (4). In the present study, we refined this methodology and applied it to obtain protein extracts of the different mycelial types: first compartmentalized mycelium (MI, 12 h); second early multinucleated mycelium (lacking the hydrophobic covers; MII, 24 h), and second late mycelium with hydrophobic layers (sporulating mycelium; MII, 72 h) (Fig. 1) (5). The MI was fractionated from the MII by obtaining protoplasts; protoplasts from multinucleated mycelium are too large to be stable. Samples of the MII can be readily obtained during the phases in which the MI has died (1, 2). Mechanical disaggregation of mycelial pellets and intensive washing removed the proteins released by the lysis of the MI (Fig. 1 and "Experimental Procedures"). Mycelial samples obtained in this way were further fractionated into three distinct subcellular fractions: cytosolic, intrinsic membrane, and extrinsic membrane proteins (Fig. 2 and supplemental Fig. 4).

Identification and Quantification of Streptomyces Proteins—The number of proteins identified and quantified in two biological replicates is reported in Fig. 3*A*. A total of 626 proteins were identified from peptide MS/MS spectra that scored above the peptide MASCOT homology threshold value (see "Experimental Procedures"). This represents 8% of the predicted *S. coelicolor* proteome. False positive peptide identification rates of 1.36 and 1.08%, respectively, were determined for the two biological replicates. A total of 359 proteins (57.3% of all the identified proteins) were detected in both biological replicates (Fig. 3*A*), and of them, 345 proteins were quantified in at least one of the developmental phases analyzed according to the criteria described in "Experimental Procedures." These 345 proteins were used in the following

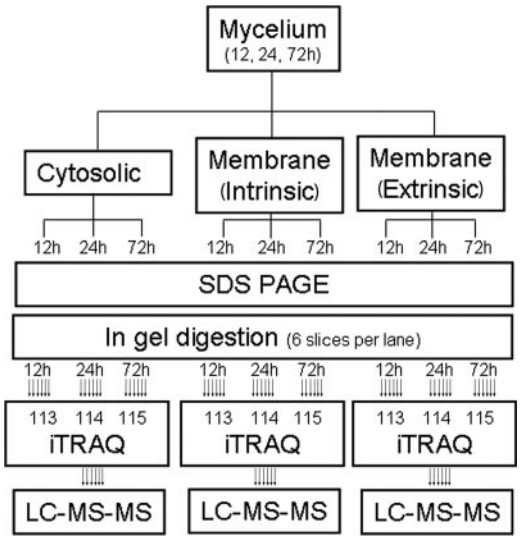


FIG. 2. **Overview of iTRAQ reagent methodology used for multiplexed comparative analysis of *Streptomyces* proteins isolated by SDS-PAGE during development.** The six steps performed in parallel for the six gel slides are indicated by *arrows*. Developmental time points and subcellular fractions are indicated.

figures, tables, and Discussion. Some proteins were identified in a single distinct cellular fraction (cytosolic, intrinsic membrane, or extrinsic membrane), whereas other proteins were detected in multiple fractions (Fig. 3*B*). The intrinsic/extrinsic membrane fractions were enriched with proteins containing at least one putative transmembrane domain or signal peptide sequences (Fig. 3*C*), demonstrating the efficiency of the fractionation strategy.

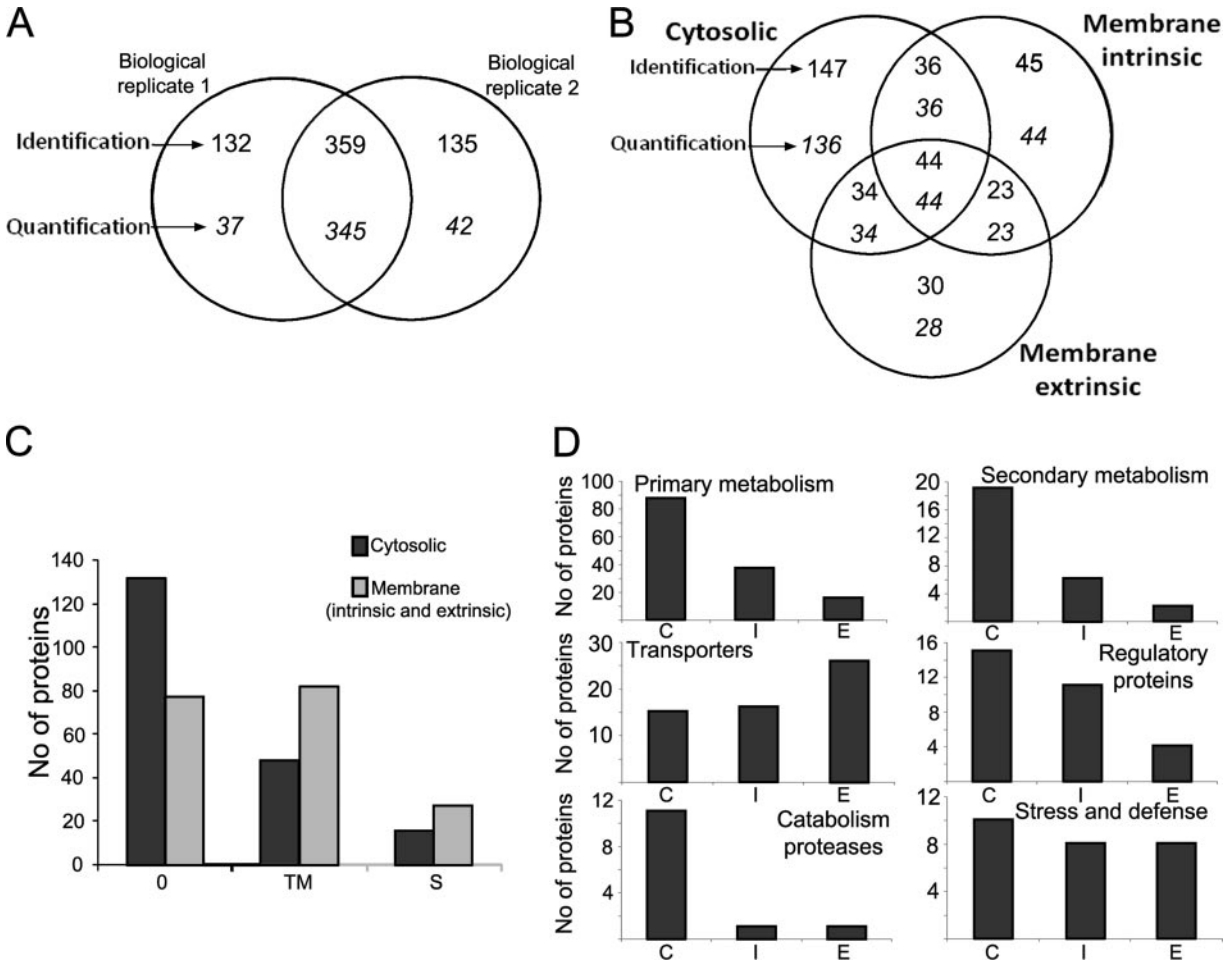


FIG. 3. **Number of identified proteins, location in subcellular fractions, and distribution in functional categories.** *A*, Venn diagram showing overlap of proteins identified in each of the two biological experiments. The number of proteins significantly quantified at least in one of the developmental phases analyzed and according to criteria described under "Experimental Procedures" is indicated. *B*, Venn diagram of protein distribution in the subcellular fractions. These proteins correspond to those detected in both biological replicates. *C*, amount of proteins in cytosol or membrane without transmembrane domain (*0*), with at least one transmembrane domain (*TM*), and with signal peptide (*S*). *D*, number of proteins for each functional category (*C*, cytosolic; *I*, membrane intrinsic; *E*, membrane extrinsic). Primary metabolism (DNA/RNA replication, aerobic and anaerobic energy production, glycolysis and glyconeogenesis, pentose phosphate pathway, amino acid metabolism, nucleotide metabolism, translation, protein folding, RNA/protein processing, and nucleases/restriction modification methylases), secondary metabolism (secondary metabolite synthesis, lipid metabolism, DNA competence, TTA BldA targets, and Bld Whi proteins), transporters (ABC transporters, transporters, and secreted proteins), regulatory proteins (transcriptional regulators, kinases, phosphatases, and other regulatory proteins), catabolism/proteases, and stress and defense are the categories shown.

Proteins were grouped according to their putative functions (Fig. 3*D* and supplemental Fig. 2). Most of the identified proteins were those involved in primary metabolism (36% of the total) and were mainly found in the cytosolic and the intrinsic membrane fractions (Fig. 3*D* and supplemental Fig. 2). Proteins related to stress (oxidative stress, chaperones, etc.), transport, and secretion processes as well as kinases were mainly found in membranes (supplemental Fig. 2).

A comparison between methodological and biological replicates of cytosolic fractions is shown in Fig. 4, *A* and *B*. The dispersion of the iTRAQ ratios of the quantified proteins was similar in the methodological and biological replicates (Fig. 4*A*). The specific iTRAQ ratio values for each protein (average of two biological replicates; see "Experimental Procedures") in a methodological replicate (Fig. 4*B*, *green line*) were within an interval of 0.1 ($\log_{10}$ iTRAQ ratio) (Fig. 4*B*, *dashed lines*), which was clearly lower than iTRAQ ratio values from biological replicates (Fig. 4*B*, *blue* and *red lines*). The correlation of the iTRAQ ratio values of all the proteins quantified in the two biological replicates (cytosolic and membrane proteins pooled together) (Fig. 4*C*) was similar to those detected for the cytosolic proteins (Fig. 4, compare *A* with *C*). The variations in logarithm of average iTRAQ ratios (from two biological replicates) for each individual protein were also similar to those for cytosolic proteins (Fig. 4, compare *A* with *D*). Variation in the iTRAQ ratios of proteins in the 72-h sample (MII)

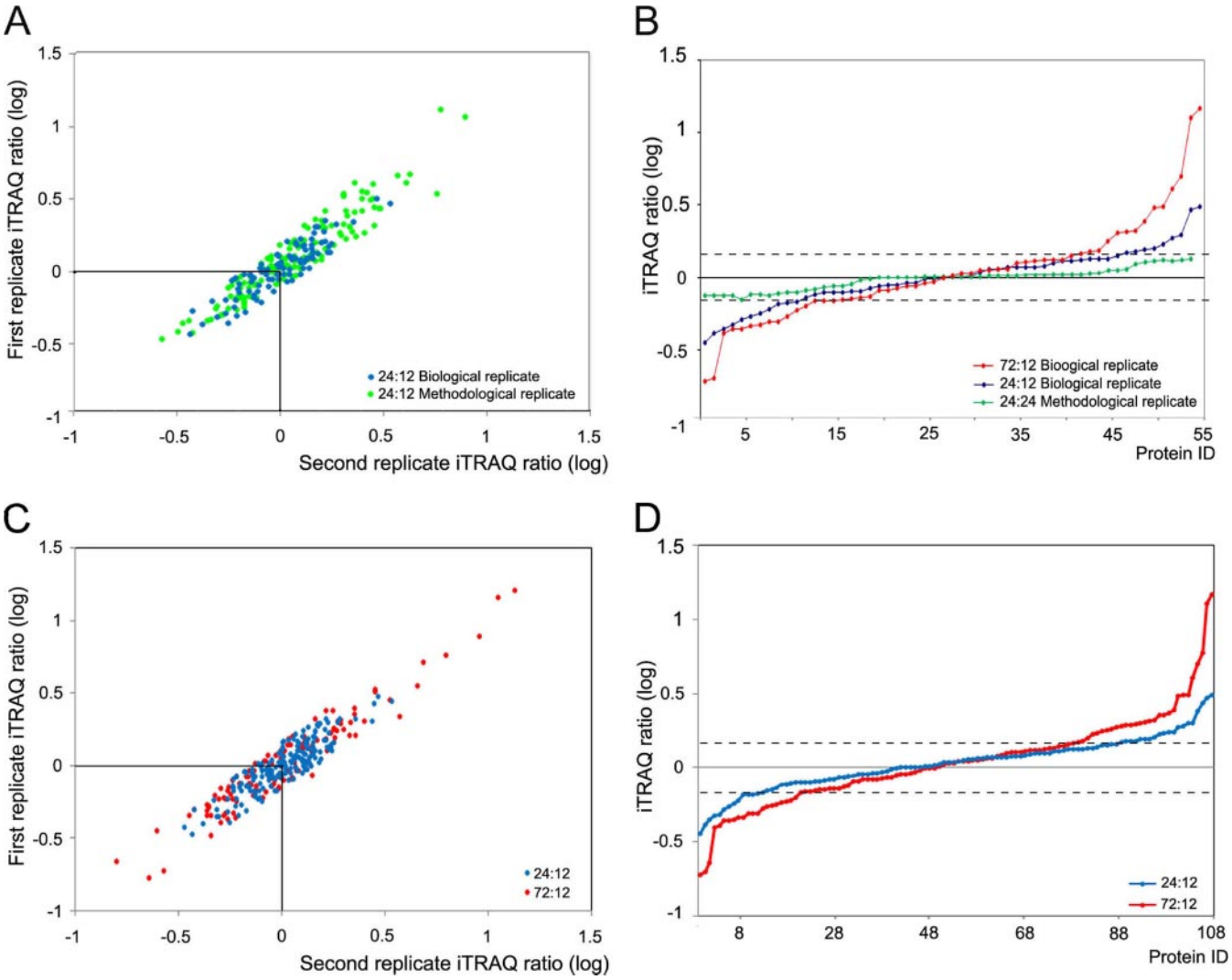
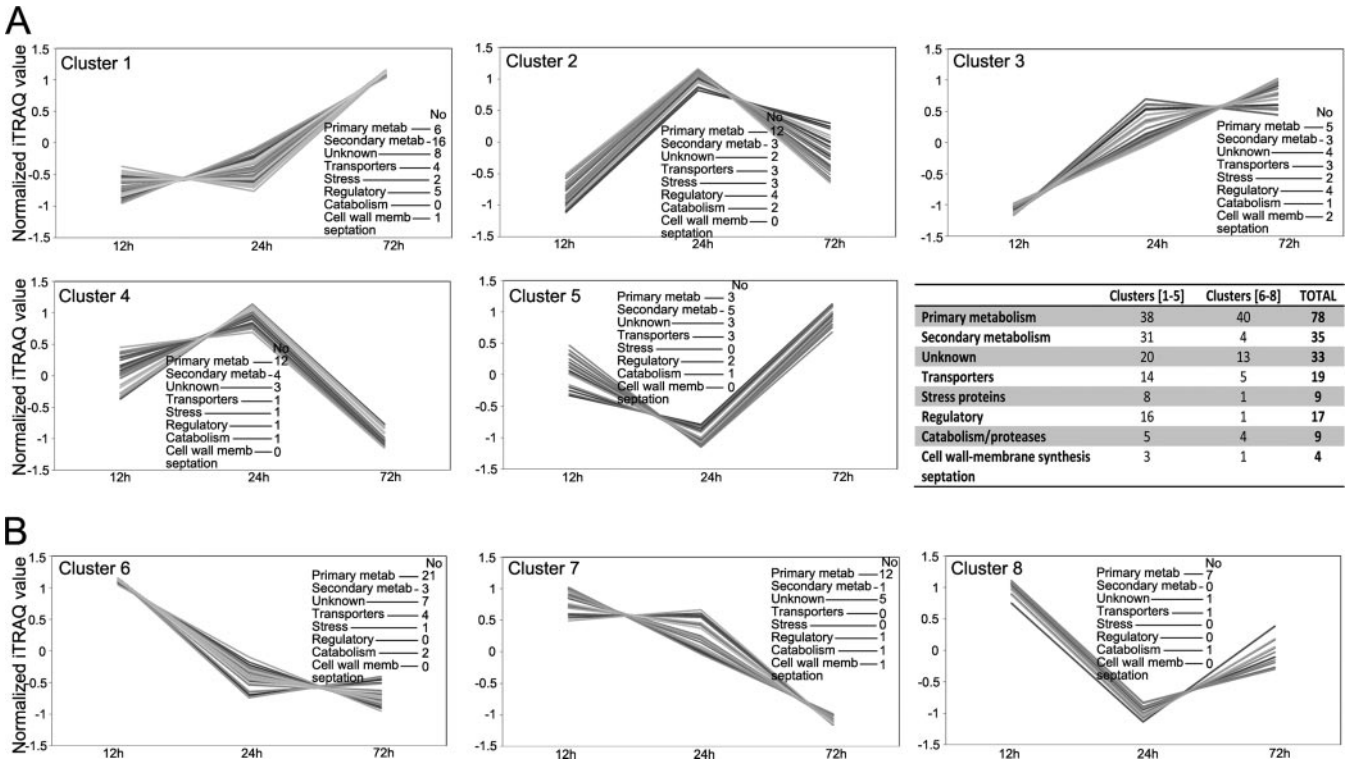


FIG. 4. **Quantitative proteomic data analysis.** *Upper panels*, comparison between methodological and biological replicates. Data from cytosolic fractions correspond to protein relative quantitation values meeting the criteria indicated under "Experimental Procedures." *A*, correlation of the values for biological replicates (*blue*) and methodological replicates (*green*). *B*, variation of iTRAQ ratios (averages from two biological replicates) in different developmental phases (*blue* and *red*) with respect to methodological variation (*green*); proteins significantly quantified in both conditions analyzed are shown (55 proteins). iTRAQ ratio values are sorted in increasing order. *Lower panels*, comparison between all the quantified proteins present in biological replicates. Cytosolic, intrinsic membrane, and extrinsic proteins were combined. *C*, correlation of the values for biological replicates: MII/24h with respect to MI/12h (*blue*) and MII/72h with respect to MI/12h (*red*). *D*, iTRAQ ratio values (averages from two biological replicates) for each protein in increasing order (108 proteins).

was greater than in the 24-h (MII) mycelial samples in both cases with respect to the 12-h sample (MI) (Fig. 4*D*).

Proteins Detected in Greater Abundance in First Compartmentalized Mycelium (MI/12h)—It is obvious from Fig. 5 (clusters 6–8) that the most abundant proteins in MI compared with the MII were those involved in primary metabolism (Table I). When proteins were grouped into functional categories (Fig. 6), this aspect is even clearer (Fig. 6, *green* colors). Examples of these proteins were ribosomal proteins (SCO4653, SCO4711, and SCO3124), glycolytic and tricarboxylic acid cycle enzymes (SCO5423, SCO2951, SCO4809, and SCO4855), enzymes involved in amino acid metabolism (SCO2504, SCO1773, and SCO3304), etc. (Table I).

Some regulatory proteins were detected in greater abundance in the MI (Table II). One of the most interesting proteins was SCO1691, a putative TetR transcriptional regulator of unknown function, that was detected exclusively in the MI (Table II). Other putative regulatory proteins with greater abundance in the MI were SCO3907, a hypothetical protein belonging to the single-stranded DNA-binding proteins with "oligonucleotide/oligosaccharide binding folds" (SSB_OBF family); SCO5537, a Ras-like GTPase; SCO3404, an FtsH homolog belonging to the "ATPase associated with a variety of cellular activities" protein family (AAA ATPases); and SCO2592, a RfaG glycosyltransferase (Table II). BldkB (SCO5113) and BldkD (SCO5115), two components of the



	Clusters [1-5]	Clusters [6-8]	TOTAL
Primary metabolism	38	40	78
Secondary metabolism	31	4	35
Unknown	20	13	33
Transporters	14	5	19
Stress proteins	8	1	9
Regulatory	16	1	17
Catabolism/proteases	5	4	9
Cell wall-membrane synthesis septation	3	1	4

FIG. 5. **Clusters of proteins detected in only one subcellular fraction (cytosolic or membrane proteins) with similar expression patterns along developmental time points.** *A*, proteins in greater abundance in MII/24h and MII/72h with respect to the MI (12 h). *B*, clusters of proteins in lower abundance in MII/24h and MII/72h with respect to MI. Numbers of proteins for each functional category are indicated. Primary metabolism (DNA/RNA replication, aerobic and anaerobic energy production, glycolysis and glyconeogenesis, pentose phosphate pathway, amino acid metabolism, nucleotide metabolism, translation, protein folding RNA/protein processing, and nucleases/RM methylases), secondary metabolism (secondary metabolite synthesis, lipid metabolism, DNA competence, TTA BldA targets, and Bld Whi proteins), proteins of unknown function, transporters (ABC transporters, transporters, and secreted proteins), stress and defense proteins, regulatory proteins (transcriptional regulators, kinases, phosphatases, and other regulatory proteins), catabolism/proteases, and cell wall/membrane/septation (cell division and septation proteins and proteins involved in cell wall and membrane synthesis) are the categories shown. Proteins clustered were those significantly quantified at least in one of the developmental phases analyzed (see "Experimental Procedures"). *metab*, metabolism; *memb*, membrane.

BldK ABC transporter complex (Table II), were also more abundant in the MI.

Proteins Detected in Greater Abundance in Second Multinucleated Mycelia (MII/24h and MII/72h)—Almost all the proteins involved in secondary metabolism were detected in greater abundance during the MII stages (MII/24h and MII/72h) than in MI (protein clusters 1–5 in Fig. 5*A* and Fig. 6): for instance, ActVA (SCO5077), ActVA4 (SCO5079), and hydroxyacyl-CoA dehydrogenase (SCO5072), all involved in the synthesis of actinorhodin, and a transketolase involved in the biosynthesis of ansamycins (SCO6663) (Table I). With a few exceptions, the absolute iTRAQ ratio values for each protein were greater at 72 h than at 24 h (Fig. 6). For example, the ratios for ActVA (SCO5077) were 8.4-fold in MII/72h and only 2.3-fold in MII/24h (Table I). Several regulatory proteins were also more abundant in MII/24h and MII/72h than in the MI (Table II). BldG (SCO3549), a transcriptional regulator that constitutes one of the latter steps of the bald cascade that is involved in *S. coelicolor* sporulation (33), was more abundant in the intrinsic membrane fraction of both MII stages (4-fold). In contrast, its abundance in the cytosol was the same in the MI and MII/24h, but its abundance was lesser in the MII/72h (Table II and Fig. 7). Other putative regulatory proteins detected in greater abundance in the MII/24h and MII/72h than in MI were SCO1793, Spo0M-homologous protein; SCO6005, a putative ABC transporter; PspA (SCO2168), phage shock protein regulating transcription; and SCO2567, ComE homolog (protein involved in the uptake of transforming DNA in Gram-positive bacteria).

Several regulatory proteins were detected in greater relative abundance in the MII/24h: SCO0168, possible cyclic nucleotide-binding transcriptional regulator; SigH (SCO5243); BldH (SCO2792); SCO2110, eukaryotic-type serine/threonine kinase; and SCO7399, putative ABC transporter (Table II).

Proteins involved in the aerobic energy production (ATP synthase chain, SCO5368) and in the glycerolipid metabolism (glycerol kinase, SCO1660) were detected in greater abundance in the MII/72h samples compared with the rest of the mycelial stages (Table I). SCO2380, a putative β-lactamase, was also more abundant in the MII/72h. Several regulatory

TABLE I

Summary of quantitative data: proteins of central metabolism (primary and secondary)

Averaged iTRAQ ratios from two biological replicates for the second multinucleated mycelial stages (MII/24h and MII/72h) with respect to the first compartmentalized mycelium (MI/12h) are shown. MII_{72h}/MII_{24h} ratios are also indicated. iTRAQ ratio values correspond to proteins significantly quantified in both early and late MII phases of *Streptomyces* development with respect to the MI (the same proteins showed in Fig. 6). Functions are according to GenBank, gene ontology, conserved domain, and KEGG. CI, clusters of proteins with similar abundances (see Fig. 5); C, cytosolic; I, membrane intrinsic; E, membrane extrinsic. KEGG metabolic pathways are as follows: 1, metabolic pathways; 2, glycolysis/gluconeogenesis; 3, purine metabolism; 4, pyruvate metabolism; 5, two-component system; 6, pentose phosphate pathway; 7, glutathione metabolism; 8, citrate cycle (tricarboxylic acid cycle); 9, oxidative phosphorylation; 10, benzoate degradation via CoA ligation; 11, butanoate metabolism; 12, propanoate metabolism; 13, C_5-branched dibasic acid metabolism; 14, glyoxylate and dicarboxylate metabolism; 15, aminoacyl-tRNA biosynthesis; 16, cysteine and methionine metabolism; 17, selenoamino acid; 18, lysine biosynthesis; 19, alanine, aspartate, and glutamate metabolism; 20, taurine and hypotaurine metabolism; 21, phenylalanine, tyrosine, and tryptophan biosynthesis; 22, arginine and proline metabolism; 23, porphyrin and chlorophyll metabolism; 24, sulfur metabolism; 25, nitrogen metabolism; 26, nitrogen metabolism; 27, glycerolipid metabolism; 28, streptomycin biosynthesis; 29, inositol phosphate metabolism; 30, pyrimidine metabolism; 31, nicotinate and nicotinamide metabolism; 32, ribosome; 33, biosynthesis of type II polyketide products; 34, biosynthesis of ansamycins; 35, ABC transporters; 36, DNA replication; 37, mismatch repair; 38, homologous recombination. See supplemental Tables 1–4 for details.

Function and KEGG	CI	SCO no.	Description	MII_{24h}/MI_{12h}			MII_{72h}/MI_{12h}			MII_{72h}/MII_{24h}		
				C	I	E	C	I	E	C	I	E
Glycolysis												
1, 2, 3, 4	8	SCO5423	Pyruvate kinase	0.8			0.8			1.1		
4, 5	7	SCO4209	Phosphoglyceromutase	1			0.7			1		
4, 5	4	SCO1946	Phosphoglycerate kinase	1.1			0.8			0.7		
Pentose phosphate pathway												
7	6	SCO4914	Deoxyribose-phosphate aldolase		0.5			0.4			0.5	
1, 6, 7	4	SCO3877	6-Phosphogluconate dehydrogenase-like	1.1			0.9			0.7		
1, 6, 7	2	SCO6661	Glucose-6-phosphate 1-dehydrogenase	1.2			1.2			0.9		
1, 6, 7	4	SCO6658	6-Phosphogluconate dehydrogenase-like	1.3			0.7			0.6		
1, 6	2	SCO6662	Transaldolase	1.4			1.2			0.8		
1, 6	2	SCO6659	Glucose-6-phosphate isomerase	2			1.1			0.6		
Krebs cycle and energy metabolism												
	6	SCO3092	NADH dehydrogenase		0.4			0.4			0.9	
1, 8, 9, 10, 11	8	SCO4855	Succinate dehydrogenase	0.6			0.8			1.3		
4, 5	6	SCO2951	Malate oxidoreductase	0.7			0.7			1		
8, 12, 13, 1	6	SCO4809	Succinyl-CoA synthetase	0.7			0.7			0.9		
1, 9	1	SCO5368	ATP synthase C chain	0.9			4.9			5.6		
1, 8, 14	2	SCO2736	Type II citrate synthase	3.1			1.2			0.4		
Nitrogen and amino acid metabolism												
15	8	SCO3304	Arginyl-tRNA synthetase	0.5			0.6			1.2		
15	6	SCO2504	Glycyl-tRNA synthetase	0.6			0.5			0.8		
1, 16, 17	7	SCO3023	*S*-Adenosyl-L-homocysteine hydrolase	0.7			0.4			0.5		
1, 18	6	SCO5739	Dihydrodipicolinate reductase	0.7			0.6			0.9		
19, 20	7	SCO1773	L-Alanine dehydrogenase	0.8			0.6			0.7		
1, 21, 22	6	SCO1494	3-Dehydroquinate synthase	0.8			0.7			0.9		
1, 15, 23	2	SCO5547	Glutamyl-tRNA synthetase	1			1			1		
16, 17, 24	4	SCO4958	Cystathionine γ-synthase		1.1			0.7			0.6	
5, 25	2	SCO0216	Nitrate reductase α chain		1.5			1.3			0.9	
1, 21, 24	1	SCO5212	3-Phosphoshikimate 1-carboxyvinyltransferase	1.3			2.1			1.6		
1, 5, 19, 22, 26	3	SCO2198	Glutamine synthetase	1.9			2.1			1.1		
Lipid metabolism												
1, 27	1	SCO1660	Glycerol kinase	1			1.5			1.5		
1, 10, 11	1	SCO3834	3-Hydroxyacyl-CoA dehydrogenase	1.5			12.7			8.4		
1, 10, 11	1	SCO5385	3-Hydroxybutyryl-CoA dehydrogenase	1.6			5.1			3.2		
Nucleotide metabolism												
1, 3	6	SCO1514	Adenine phosphoribosyltransferase	0.6			MI[a]			MII_{24h}[a]		
1, 28, 29	6	SCO3899	*myo*-Inositol-1-phosphate synthase	0.6			0.5			0.9		
1, 3	7	SCO3060	Phosphoribosylaminoimidazole carboxylase	0.8			0.5			0.6		
1, 3, 30, 31	7	SCO4917	Purine-nucleoside phosphorylase	1			0.7			0.7		

TABLE I—*continued*

Function and KEGG	CI	SCO no.	Description	MII_{24h}/MI_{12h}			MII_{72h}/MI_{12h}			MII_{72h}/MII_{24h}		
				C	I	E	C	I	E	C	I	E
1, 27	1	SCO1660	Glycerol kinase	1			1.5			1.5		
1, 3	1	SCO4087	Purine metabolism	1.4			2.2			1.5		
3, 19	3	SCO3629	Adenylosuccinate synthetase	1.6			1.5			0.9		
Translation, protein folding, RNA/protein processing												
32	6	SCO4711	30 S ribosomal protein S17	0.3			0.2			0.6		
32	7	SCO4653	50 S ribosomal protein L7/L12	0.4			0.2			0.8		
32	6	SCO4735	30 S ribosomal protein S9	0.4			0.4			0.9		
32	6	SCO4718	50 S ribosomal protein L18	0.4			0.4			1		
32	6	SCO3124	50 S ribosomal protein L25	0.5			0.3			0.6		
	8	SCO2620	Trigger factor	0.5			0.6			1.2		
	8	SCO5221	Polypeptide deformylase	0.5			0.7			1.3		
	6	SCO5625	Elongation factor Ts	0.5			0.5			1		
32	6	SCO4710	50 S ribosomal protein L29	0.5			0.4			0.8		
32	6	SCO4719	30 S ribosomal protein S5	0.5			0.4			0.8		
32	6	SCO4721	50 S ribosomal protein L15	0.5			0.4			0.8		
32	6	SCO3909	50 S ribosomal protein L9	0.5			0.5			0.9		
	6	SCO3970	Xaa-Pro aminopeptidase	0.6			0.5			0.9		
32	7	SCO4713	50 S ribosomal protein L24	0.9			0.6			0.6		
	7	SCO3874	DNA topoisomerase		0.9			0.8			0.9	
	7	SCO2562	GTP-binding protein LepA	1			0.8			0.8		
	2	SCO1600	InfC translation initiation factor IF-3			1.1			0.7			0.6
32	3	SCO4702	50 S ribosomal protein L3	1.2			1.3			1.1		
	2	SCO1491	Elongation factor P	1.8			1.2			0.7		
Secondary metabolite synthesis												
	5	SCO2380	β-Lactamase	0.8			2.5			3.2		
33	1	SCO5086	Ketoacyl reductase		1.1			1.9			1.7	
33	1	SCO5072	Hydroxyacyl-CoA dehydrogenase		1.2			1.5			1.2	
33	1	SCO5079	ActVA4		1.2			2.1			1.7	
	1	SCO0395	Epimerase/dehydratase		1.3			1.9			1.5	
1, 6, 34	2	SCO6663	Transketolase		1.4			1.3			0.9	
33	1	SCO5077	ActVA	2.3			8.4			3.7		
Degradative enzymes: nucleases, proteases												
	6	SCO5745	mRNA degradation ribonucleases J1/J2	0.7			0.7			1.1		
	4	SCO2793	Oligoribonuclease; 3′–5′-exoribonuclease	1.1			0.7			0.7		
	1	SCO1230	Putative secreted tripeptidyl aminopeptidase	1.6			28.1			17.8		

[a] Proteins detected only in MI and MII_{24h}.

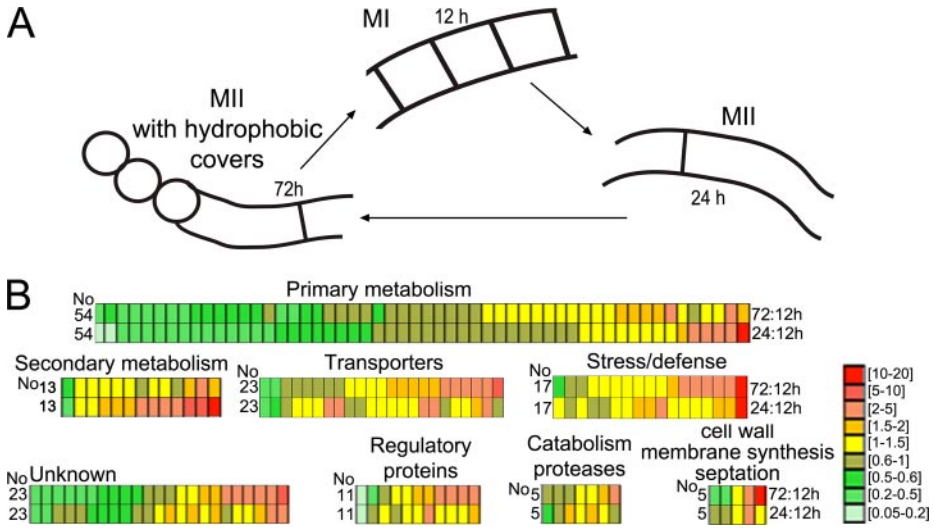


FIG. 6. **iTRAQ ratios values for proteins grouped into functional categories.** *A*, scheme of *Streptomyces* developmental cycle. Different mycelial phases and developmental time points are schematically represented. MI, first compartmentalized mycelium. MII, second multinucleated mycelia (early and late phases). *B*, iTRAQ ratios for proteins significantly quantified in both MII/24h and MII/72h of *Streptomyces* development with respect to MI grouped into the functional categories indicated in Fig. 5. The total number of proteins is indicated on the *left* of the panels. Cytosolic and membrane proteins are pooled together.

proteins were detected in greater abundance at 72 h: SCO5046, a WhiB family σ factor; SCO5580, FtsY docking protein; SCO1630 and SCO4677, histidine kinases; SCO4920, transcriptional regulator; SCO3571 and SCO1648, ARC AAA ATPases; SCO5249, a CAP family transcriptional regulator; and SCO5466, a putative autolytic lysozyme that could have a role during sporulation (Table II).

Abundance of Proteins Detected in More Than One Subcellular Fraction—One hundred and forty proteins were detected in more than one subcellular fraction (supplemental Table 4 and Fig. 7). As in the case of proteins detected only in one subcellular fraction (Fig. 6), with few exceptions, the proteins more abundant in MII/24h were also more abundant in MII/72h (relative to MI/12h), and the same was found for the less abundant proteins (supplemental Table 4). Some of these proteins showed different iTRAQ ratios among cellular compartments, suggesting a change of subcellular location during *Streptomyces* differentiation (Fig. 7). Some examples of these proteins were SCO1965, a putative stress protein (without transmembrane domains); SCO4296, chaperonin GroEL (two transmembrane domains); SCO2231 (one signal peptide), a putative bacterial transport protein; and SCO3549, BldG (see above; MII proteins).

DISCUSSION

Experimental initiatives to perform proteomics and transcriptomics analyses in *Streptomyces* have been hampered because of the lack of a reliable developmental model in this bacterium. One of the main drawbacks has been the use of samples in which different mycelial structures and phases coexist. In the present work, we overcame the problem by means of sample fractionation based on the different developmental phases and types of mycelium recently described for *Streptomyces* (MI/12h, MII/24h, and MII/72h) (1–6) (Fig. 1). By using iTRAQ labeling and LC-MS/MS, we were able to identify a total of 626 proteins (8% of *S. coelicolor* proteome) in at least one of the two biological replicates analyzed. Three hundred and fifty-nine of these proteins were detected in both biological replicates (4.6% of the proteome), and 345 (4.4% of the proteome) were quantified with high confidence (Fig. 3). The MI proteome was particularly rich in primary metabolism proteins, whereas the MII proteome was enriched in proteins involved in secondary metabolism, stress, defense, and transport (Table I and Figs. 5 and 6). Overall, the switching on of secondary metabolism correlates with hypha differentiation. Knowledge of the mycelial stage involved in secondary metabolism is an important aspect of *Streptomyces* biology that will open new perspectives in the experimentation with this bacterium, including submerged cultures in which there is no sporulation, but the MII stage exists (7). These aspects will have repercussions in industrial fermentations where conditions that allow hypha differentiation have been largely ignored (7).

S. coelicolor A3(2) developmental mutants have been useful for the genetic and biochemical analysis of the differentiation cycles. The so-called "bald" (*bld*) mutants (considered defective in aerial growth) (34, 35) and "white" (*whi*) mutants (defective in the formation of mature gray spores on the fluffy aerial mycelium) (36, 37) fail to complete normal development. The *bld* genes control the onset of aerial hypha formation by regulating the expression of genes involved in the production of SapB (38–41), rodlins (42), and chaplins (43). In this work, proteins encoded by these genes were identified and quantified. The BldK complex, implicated in the initiation of the bald signaling network, is a well known oligopeptide transporter that acts as a differentiation signal for *S. coelicolor* (44, 45). Two components of the Bldk ABC transporter complex (BldkB and BldkD) were quantified in lesser abundance in the MII than in the MI (Table II), consistent with a role in the early development. σ factor H (SCO5243) is another well characterized regulatory protein whose gene expression has been described coinciding with the onset of aerial mycelium formation (47). SigH was more abundant in the cytosolic fraction of

TABLE II

Summary of quantitative data: putative regulatory proteins

Averaged iTRAQ ratios from two biological replicates for the second multinucleated mycelial stages (MII/24h and MII/72h) with respect to the MI are shown. MII_{72h}/MII_{24h} ratios are also indicated. C, cytosolic; I, membrane intrinsic; E, membrane extrinsic. Eukaryotic-type signaling domains are indicated as well as the protein functions of well characterized proteins. n.s., non-significant iTRAQ ratio value (see "Experimental Procedures"). See supplemental Tables 1–4 for details. Functions, clusters (Cl), and KEGG pathways are as in Table I. Superscript AP indicates eukaryotic-like AP ATPases. Superscript K indicates eukaryotic-type Ser/Thr kinases.

Function and KEGG	Cl	SCO no.	Description	MII_{24h}/MI_{12h}			MII_{72h}/MI_{12h}			MII_{72h}/MII_{24h}		
				C	I	E	C	I	E	C	I	E
Cell division/septation												
	7	SCO5537	Ras-like GTPase	0.79			0.49			0.62		
		SCO3404	Cell division protein FtsH	0.85	0.98		0.88	n.s.		1.03	n.s.	
		SCO5580^{AP}	FtsY docking protein	n.s.			2.2			n.s.		
	3	SCO1793	Spo0M-homologous protein	2.17			2.77			1.28		
TTA BldA targets and Bld Whi proteins												
35		SCO5115[a]	BldkD		n.s.	0.49		0.78	0.89		n.s.	1.81
35		SCO5113[a]	BldkB	1.05	n.s.	0.65	0.95	0.73	n.s.	0.9	n.s.	n.s.
35		SCO3958^{AP}	ABC transporter ATP-binding protein		0.83	0.89		n.s.	1.21		n.s.	1.35
	1	SCO5046	WhiB family σ factor	0.98			2.02			2.06		
		SCO2792[a]	AdpA (BldH)		n.s.	1.84		n.s.	n.s.		n.s.	n.s.
		SCO3549[a]	BldG	1.04	4.73		0.63	4.79	0.6	1.01		
Transcriptional regulators												
		SCO3571	Transcriptional regulator	1	1.18		1	2.02		1	1.71	
	3	SCO2168[a]	PspA transcriptional regulator			1.33			1.98			1.49
	1	SCO4920	DeoR transcriptional regulator		1.46			2.1			1.44	
	2	SCO5243[a]	σ factor H	2.17			n.s.			n.s.		
	2	SCO0168	Cyclic nucleotide-binding transcriptional regulator	2.95			1.52			0.51		
		SCO5249	CAP family transcriptional regulator	n.s.	1.09	1.22	9.7	2.38	2.69	n.s.	2.18	2.2
		SCO1691	TetR transcriptional regulator	MI[b]			MI[b]					
Kinases												
	4	SCO2110^{K}	Eukaryotic-type serine/threonine kinase		1.33			0.91			0.68	
		SCO1630	Histidine kinase		1.38	n.s.		3.68	2.16	2.67	n.s.	
	3	SCO4677	Histidine kinase		1.82			3.15			1.73	
Cell wall synthesis/degradation/ division/septation												
		SCO2592	RfaG glycosyltransferase	1.13	0.81	0.89	1.3	0.49	0.32	1.15	0.6	0.36
	1	SCO5466	Putative autolytic lysozyme	1.17			14.7			12.6		
	5	SCO2567	ComE homolog protein			1.48			2.14			1.44
Other regulatory proteins												
36, 37, 38	7	SCO3907	DNA-binding protein	0.79			0.46			0.58		
	3	SCO1648	ARC AAA ATPase		n.s.			1.36			n.s.	
ABC transporters												
	6	SCO1840^{AP}	ABC transporter ATP-binding protein		0.47			0.47			1	
35	6	SCO1559	ABC MetN methionine transporter		0.65			0.45			0.69	
		SCO2677^{AP}	ABC transporter ATP-binding protein	0.65	0.62		n.s.	0.55			0.89	
35	5	SCO5480	Oligopeptide ABC transporter			0.7			n.s.			n.s.
35	3	SCO6005	ABC transporter			2.51			4.60			1.83
		SCO7399	ABC transporter system	3.1	2.39	2.69	2.12	0.89	1.46	0.68	0.37	0.54

[a] Well characterized proteins.

[b] Proteins detected exclusively in first compartmentalized mycelium.

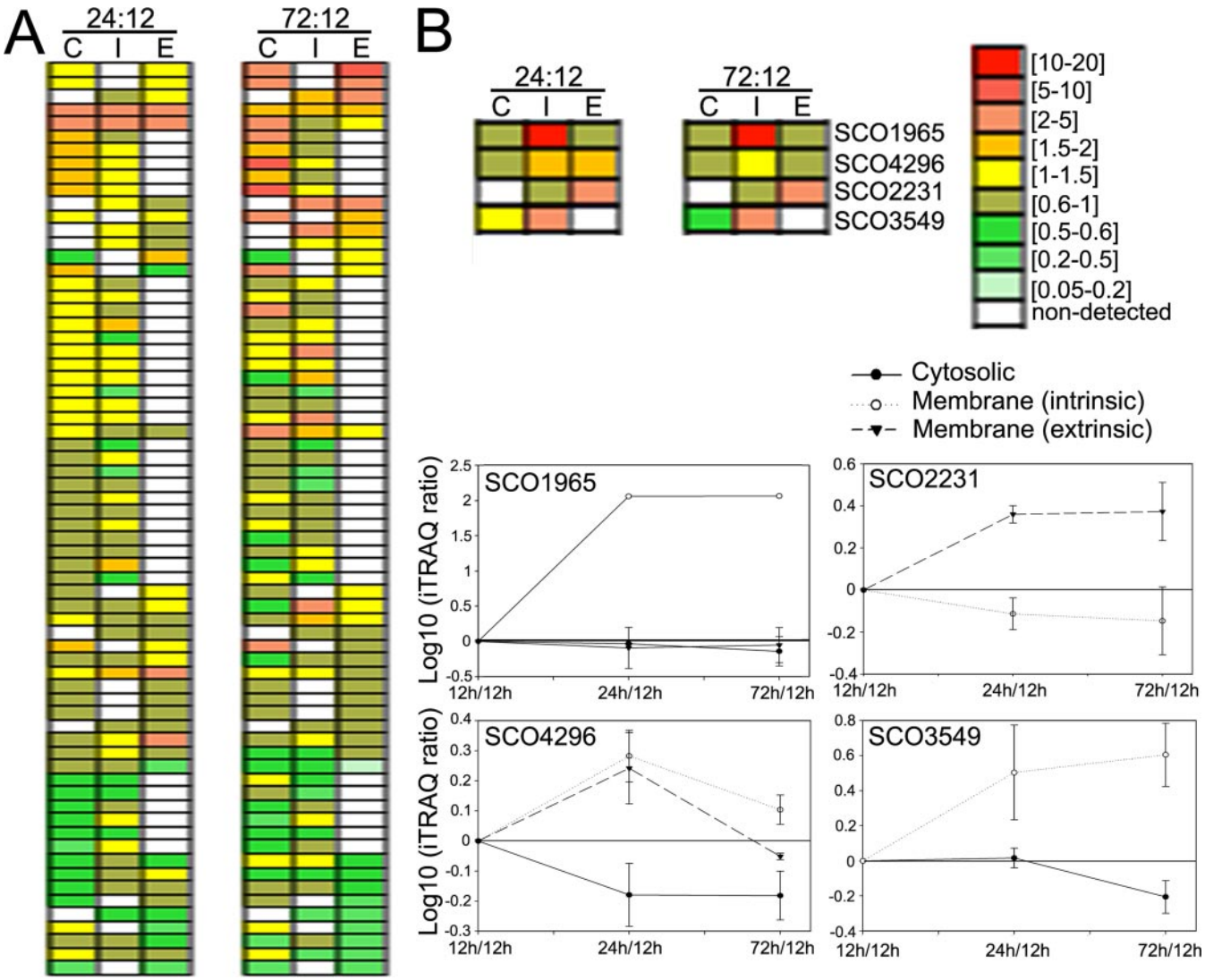


FIG. 7. **Proteins detected in more than one subcellular fraction.** *A*, iTRAQ ratios for proteins identified in more than one subcellular fraction. Values are the average of the two biological replicates analyzed meeting the reproducibility criteria described under "Experimental Procedures." *C*, cytosolic; *I*, membrane intrinsic; *E*, membrane extrinsic. *B*, proteins with different abundance values between subcellular fractions. *Error bars* correspond to the S.D. See text for details.

the MII/24h, indicating that this mycelium was already involved in a differentiation process. This result was consistent with some reports describing an "ectopic expression" of the *sigH* gene in the substrate mycelium of *bldD* mutant strains (48). BldH (AdpA, SCO2792) is an AraC-like protein considered a "master regulator" through which *bldA* exerts its effects on differentiation and secondary metabolism (for a review, see Ref. 46). It was more abundant (1.84-fold; Table II) in the MII/24h with respect to the rest of the mycelial types, confirming its role in the developmental phases preceding the formation of hydrophobic covers (19). BldG (SCO3549) is a transcriptional regulator involved in one of the latter steps of the bald cascade and *S. coelicolor* sporulation (33). Its expression was similar in MII/24h and MII/72h (Table II), supporting that these mycelial types share similar differentiation processes. SCO5046 is a WhiB family σ factor regulating the final sporulation steps (26, 37). This protein was more abundant (2.02-fold; Table II) in MII/72 than in MI and MII/24h, consistent with its role in sporulation and hydrophobic cover formation. In short, the expression pattern of all these developmental proteins confirmed that MII/24h is already involved in a differentiation process and is functionally distinct from MI. The main differences between the second mycelial types resided in the proteins involved in hydrophobic cover formation and the final stages of sporulation (AdpA, BldK, and WhiB) but not in the proteins regulating physiology and early stages of sporulation (BldG and SigH transcriptional regulators).

SCO1230 (putative tripeptidyl aminopeptidase; 28-fold), SCO3834 (lipid metabolism; 13-fold), SCO5385 (lipid metab-

olism; 5-fold), SCO5368 (ATP synthase C chain; 5-fold), and SCO5466 (putative autolytic lysozyme; 15-fold) were detected in greatest abundances in the MII/72h with respect to the other developmental stages. SCO1230, SCO3834, and SCO5385 are degradative proteins, which could be involved in an extensive degradation of some cellular constituents (proteins and lipids) accompanying antibiotic production. The overexpression of the ATP synthase (SCO5368) might indicate that several reduced cofactors are being produced and thus that somehow central metabolism is likely to be very active. SCO5466 shows homologies with *Clostridium* autolytic lysozymes and could be involved in the reorganization of the cell wall accompanying hypha fragmentation into spores. In addition, several uncharacterized regulatory proteins were detected as being differentially expressed during the *Streptomyces* hypha differentiation process (Table II). The biological functions of many of these proteins are not characterized in *Streptomyces*, and further work will be necessary to determine their role in differentiation.

Some proteins were detected in more than one subcellular fraction (supplemental Table 4 and Fig. 7), which might be explained by cross-contamination. However, the fact that these proteins are a minority (38% of all the identified proteins) (Fig. 3), that their relative abundances were similar in both MII stages and different in MI, and that some of them showed different iTRAQ ratios among cellular compartments suggested that this is not the case (Fig. 7). Moreover, some of these proteins have been described in other organisms as present in more than one subcellular fraction. For instance, SCO1965 belongs to the TerD family of proteins, which may be associated with protein export systems (49); SCO4296 belongs to the GroEL type chaperonins, which are present in the cytosol but also interact with membranes (50).

In summary, the highly detailed and comprehensive quantitative proteomic analysis of the *S. coelicolor* M145 differentiation stages presented here constitutes the most complete database of protein profiles during *Streptomyces* development in solid cultures described to date. Several proteins were detected as being differentially expressed during development, and their detailed genetic and biochemical analyses will undoubtedly provide valuable information on *Streptomyces* differentiation processes in the future.

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