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

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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...
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 × 10² 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 MgCl2, 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; 10 Petri dishes at 24 h and 10 plates at 72 h) precooled at 0 °C and centrifuged for 10 min at 5000 × 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 × 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 × 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 × g. This process was repeated three times, discarding the supernatants. Membranes were later resuspended in 100 mM Na2CO3 (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 Na2CO3 (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 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, MI/24h, and MI/72h) of each subcellular fraction and the two biological replicates were loaded in six different gels, which were used for six independent iTRAQ triplet 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 C18 (Empore extraction disks, 3M) and R3 material (see Fig. 2).
**Streptomyces Proteome Variations during Differentiation**

**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-μm PEEKsil sample loop (SGE Analytical Science, Darmstadt, Germany). Mobile phase A was 0.1% formic acid in double distilled H2O, 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 C18 column (1.7 μm, 15-cm × 75-μm analytical reverse 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, 0 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, MI/24h, and MI/72h) of each subcellular fraction and each biological replicate were processed independently. Consequently, we performed six independent iTRAQ tripex 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 (± 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 ± 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 log2-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 c, 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-
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 and late multinucleated mycelia (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. 3A. 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. 3A), 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 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. 3B). The intrinsic/extrinsic membrane fractions were enriched with proteins containing at least one putative transmembrane domain or signal peptide sequences (Fig. 3C), demonstrating the efficiency of the fractionation strategy.
Proteins were grouped according to their putative functions (Fig. 3D 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. 3D 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. 4A). The specific iTRAQ ratio values for each protein (average of two biological replicates; see “Experimental Procedures”) in a methodological replicate (Fig. 4B, green line) were within an interval of 0.1 (log10 iTRAQ ratio) (Fig. 4B, dashed lines), which was clearly lower than iTRAQ ratio values from biological replicates (Fig. 4B, 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. 4C) 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)
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 “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

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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).
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. 5A 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
Averaged iTRAQ ratios from two biological replicates for the second multinucleated mycelial stages (MI/24h and MI/72h) with respect to the first compartmentalized mycelium (MI/12h) are shown. MI/72h/MI/24h ratios are also indicated. iTRAQ ratio values correspond to proteins significantly quantified in both early and late MI 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. Cl, 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 (TCA cycle); 9, oxidative phosphorylation; 10, benzoate degradation via CoA ligation; 11, butanoate metabolism; 12, propionate metabolism; 13, C3-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.

### Table I

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

| Function and KEGG | Cl | SCO no. | Description | MI/24h/MI/12h | MI/72h/MI/12h | MI/72h/MI/24h |
|-------------------|----|---------|-------------|--------------|--------------|--------------|
| Glycolysis        | 1, 2, 3, 4 | 8 | SCO0543 | Pyruvate kinase | 0.8 | 0.8 | 1.1 |
|                   | 4, 5 | 7 | SCO4206 | Phosphoglyceromutase | 1 | 0.7 | 1 |
|                   | 4, 5 | 4 | SCO1946 | Phosphoglycerate kinase | 1.1 | 0.8 | 0.7 |
| Pentose phosphate pathway | 6 | SCO4914 | Deoxyxylulose-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 | SCO3078 | 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 dehydrogenase | 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, 6, 17 | 7 | SCO3023 | 3-Adenosyl-β-homocysteine hydrolase | 0.7 | 0.4 | 0.5 |
| 1, 18 | 6 | SCO5398 | Dihydrolipoyltransacetylase | 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 | Glutaryl-tRNA synthetase | 1 | 1 | 1 |
| 16, 17, 24 | 4 | SCO4958 | Cystathionine β-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 | SCO0660 | Glycerol kinase | 1 | 1.5 | 1.5 |
| 1, 10, 11 | 1 | SCO3894 | 3-Hydroxyacyl-CoA dehydrogenase | 1.5 | 1.27 | 8.4 |
| 1, 10, 11 | 1 | SCO3585 | 3-Hydroxybutyryl-CoA dehydrogenase | 1.6 | 5.1 | 3.2 |
| Nucleotide metabolism | 1, 3 | 6 | SCO1514 | Adenine phosphoribosyltransferase | 0.8 | 0.8 | 1.1 |
| 1, 28, 29 | 6 | SCO3989 | myo-Inositol-1-phosphate synthase | 0.8 | 0.5 | 0.9 |
| 1, 3 | 7 | SCO3960 | Phosphorylase b | 0.8 | 0.5 | 0.6 |
| 1, 3, 30, 31 | 7 | SCO4917 | Purine-nucleoside phosphorylase | 1 | 0.7 | 0.7 |
| Function and KEGG | Cl | SCO no. | Description | \( M_{II24h}/M_{I2h} \) | \( M_{II72h}/M_{I2h} \) | \( M_{II72h}/M_{II24h} \) |
|------------------|----|---------|--------------|----------------|----------------|----------------|
| Translation, protein folding, RNA/protein processing | 32 | 6 | SCO4711 | 30 S ribosomal protein S17 | 0.3 | 0.3 | 1 |
| | 32 | 7 | SCO4653 | 50 S ribosomal protein L7/L12 | 0.4 | 0.4 | 0.9 |
| | 32 | 6 | SCO4735 | 30 S ribosomal protein S9 | 0.5 | 0.5 | 1 |
| | 32 | 6 | SCO4718 | 50 S ribosomal protein L18 | 0.6 | 0.6 | 1 |
| | 32 | 6 | SCO3124 | 50 S ribosomal protein L25 | 0.5 | 0.5 | 1 |
| | 8 | 8 | SCO2620 | Trigger factor | 0.5 | 0.5 | 1 |
| | 6 | 6 | SCO5622 | Elongation factor Ts | 0.5 | 0.5 | 1 |
| | 32 | 6 | SCO4710 | 50 S ribosomal protein L29 | 0.5 | 0.5 | 1 |
| | 32 | 6 | SCO4719 | 30 S ribosomal protein S5 | 0.5 | 0.5 | 1 |
| | 32 | 6 | SCO4721 | 50 S ribosomal protein L15 | 0.5 | 0.5 | 1 |
| | 32 | 6 | SCO3909 | 50 S ribosomal protein L9 | 0.5 | 0.5 | 1 |
| | 32 | 6 | SCO3970 | Xaa-Pro aminopeptidase | 0.6 | 0.6 | 1 |
| | 7 | 7 | SCO4713 | 50 S ribosomal protein L24 | 0.9 | 0.9 | 1 |
| | 7 | 6 | SCO3874 | DNA topoisomerase | 0.9 | 0.9 | 1 |
| | 7 | 6 | SCO2562 | GTP-binding protein LepA | 0.8 | 0.8 | 1 |
| | 2 | 2 | SCO1600 | InIC translation initiation factor IF-3 | 0.7 | 0.7 | 1 |
| | 32 | 7 | SCO4702 | 50 S ribosomal protein L3 | 1.2 | 1.2 | 1 |
| | 32 | 2 | SCO1491 | Elongation factor P | 1.8 | 1.8 | 1 |
| Secondary metabolite synthesis | 5 | 5 | SCO2380 | β-Lactamase | 0.8 | 0.8 | 1 |
| | 33 | 1 | SCO5086 | Ketocycl reductase | 1.1 | 1.1 | 1 |
| | 33 | 1 | SCO5072 | Hydroxacyl-CoA dehydrogenase | 1.2 | 1.2 | 1 |
| | 33 | 1 | SCO5079 | ActVA4 | 1.2 | 1.2 | 1 |
| | 33 | 1 | SCO0395 | Epimerase/dehydratase | 1.3 | 1.3 | 1 |
| | 1, 6, 34 | 2 | SCO5663 | Transketolase | 1.4 | 1.4 | 1 |
| | 33 | 1 | SCO5077 | ActVA | 2.3 | 2.3 | 1 |
| Degradative enzymes: nucleases, proteases | 6 | 5 | SCO5714 | mRNA degradation ribonucleases J1/J2 | 0.7 | 0.7 | 1 |
| | 4 | 5 | SCO2733 | Oligoribonuclease; 3'-5'-exoribonuclease | 1.1 | 1.1 | 1 |
| | 1 | 5 | SCO1230 | Putative secreted tripeptidyl aminopeptidase | 1.6 | 1.6 | 1 |

* Proteins detected only in MI and \( M_{II24h} \).
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 SCO4666, a putative autolytic lysozyme that could have a role during sporulation (Table II). One hundred and forty 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 SCO4666, 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 localization 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); and SCO3571 and SCO1648, ARC AAA ATPases; SCO5249, a CAP family transcriptional regulator; and SCO4666, a putative autolytic lysozyme that could have a role during sporulation (Table II).

**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), rodlin (42), and chaplin (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. MII72h/MII24h 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 | MII24h/MI12h | MII72h/MI12h | MII72h/MII24h |
|-------------------|----|---------|-------------|-------------|-------------|--------------|
|                   |    |         |             | 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 | 0.88 | n.s. | 1.03 | n.s. | 1.03 |
|                    |     | SCO5580 | 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 | BldkD | n.s. | 0.49 | n.s. | 0.9 | n.s. | 1.81 |
|                    | 35  | SCO5113 | BldkB | 1.05 | 0.95 | 0.78 | 0.89 | n.s. | n.s. |
|                    | 35  | SCO3549 | ABC transporter ATP-binding protein | n.s. | 2.02 | 2.06 |
|                    | 1   | SCO5046 | WhiB family σ factor | 0.98 | 0.65 | n.s. | 1.03 | n.s. | 1.03 |
|                    | 3   | SCO2168 | PspA transcriptional regulator | 1.33 | 1.98 | 1 | 1.71 |
|                    | 3   | SCO4920 | DeoR transcriptional regulator | 1.46 | 2.1 | 1.44 |
|                    | 2   | SCO5243 | Histidine kinase | 2.17 | n.s. | 3.68 | n.s. | 2.67 | n.s. |
|                    | 2   | SCO0168 | Cyclic nucleotide-binding transcriptional regulator | 2.95 | 1.22 | 2.38 | n.s. | 2.18 | 2.2 |
| Kinases | 4   | SCO2110 | Eukaryotic-type serine/threonine kinase | 1.33 | 0.91 | 0.68 |
|                    | 3   | SCO1630 | Histidine kinase | 1.38 | 3.68 | 2.16 | 2.67 | n.s. | 1.73 |
| Cell wall synthesis/degradation/division/septation | 3   | SCO2592 | RfaG glycosyltransferase | 1.13 | 0.81 | 0.89 | 0.32 | 1.15 | 0.36 |
|                    | 5   | SCO5466 | Putative autolytic lysozyme | 1.17 | 1.47 | 1.26 |
|                    | 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 | ABC transporter ATP-binding protein | 0.47 | 0.47 | 1 |
|                    | 6   | SCO1559 | ABC MetN methionine transporter | 0.65 | 0.45 | 0.69 |
|                    | 5   | SCO2677 | ABC transporter ATP-binding protein | 0.65 | 0.55 | 0.89 |
|                    | 5   | SCO3549 | ABC transporter ATP-binding protein | n.s. | 1.7 | n.s. |
|                    | 3   | SCO6005 | ABC transporter | 2.51 | 4.60 | 1.83 |
|                    | 3   | SCO7399 | ABC transporter system | 3.1  | 2.39 | 2.69 | 2.12 | 0.89 | 1.46 | 0.68 | 0.37 | 0.54 |

* Well characterized proteins.
** Proteins detected exclusively in first compartmentalized mycelium.
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), SCO3835 (lipid metab-
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...will undoubtedly provide valuable information on... development, and their detailed genetic and biochemical analyses in solid cultures described to date. Several proteins database of protein profiles during... initiation stages presented here constitutes the most complete... belongs to the GroEL type chaperonins, which are present in... SCO4296 belongs to the TerD family of proteins, which may... attached with protein export systems (49); SCO4296... showed different iTRAQ ratios among cellular compartments... 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 MI 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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