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Analysis of *Streptomyces coelicolor* membrane proteome using two-dimensional native/native and native/sodium dodecyl sulfate gel electrophoresis

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ABSTRACT

Analysis of the oligomeric state of a protein may provide insights into its physiological functions. Because membrane proteins are considered to be the workhorses of energy generation and polypeptide and nutrient transportation, in this study we characterized the membrane-associated proteome of *Streptomyces coelicolor* by two-dimensional (2D) blue native/sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), high-resolution clear native/native PAGE, and native/SDS–PAGE. A total of 77 proteins were identified, and 20 proteins belonging to 15 complexes were characterized. Moreover, the resolution of high-resolution clear native/SDS–PAGE is much higher than that of blue native/SDS–PAGE. OBP (SCO5477) and BldKB (SCO5113) were identified as the main protein spots from the membrane fractions of *S. coelicolor* M145, suggesting that these two proteins are involved in extracellular peptide transportation. These two transporters exhibited multiple oligomeric states in the native PAGE system, which may suggest their multiple physiological functions in the development of *S. coelicolor*.

Streptomyces is the largest genus of Actinobacteria and includes more than 500 species. *Streptomyces* spp. are filamentous and soil-dwelling and produce more than two-thirds of clinically useful antibiotics as well as anticancer, antiparasitic, and immunosuppressant drugs [1]. *Streptomyces* spp. are morphologically and physiologically complex. *Streptomyces* colonies can develop into young foraging mycelium, mature vegetative mycelium, aerial hyphae, and spores. This development is controlled by complex regulatory circuits, an example of which is the regulation of antibiotic production and morphological development in *Streptomyces coelicolor* by the rare leucine codon UUA.

Modern genomic and proteomic techniques are being used to analyze *Streptomyces* to unravel its biology and physiology [2–5]. Two-dimensional (2D)[1] electrophoresis and protein fingerprinting techniques were used to characterize 120 gene products from the *S. coelicolor* membrane fraction, and only a few differences were found between the membrane proteome of the wild-type and mutant strains [2]. Furthermore, a combination of proteomic and transcriptomic analysis of the *bldA* strain revealed that 2% of its total genome was affected by the mutation; moreover, diverse levels of pleiotropic regulatory effects were detected, including post-translational modifications [3].

Proteins assemble into complexes to perform physiological functions. Therefore, analysis of these protein complexes can help to determine the behavior of these proteins and their cellular functions. Blue native electrophoresis (BNE) is an efficient tool for the analysis of protein complexes at the proteomic scale. Established in 1991, this method was initially used to analyze membrane protein complexes [6]. In this method, the Coomassie Brilliant Blue G-250 dye is added into the electrophoresis buffer. This anionic dye binds to the surface of the membrane proteins and facilitates their migration in the native gradient polyacrylamide gel [7]. Coupled with second-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and mass spectrometry, protein complexes from animals, plants, and bacteria were efficiently characterized [8–10].

A modified native PAGE, termed high-resolution clear native electrophoresis (hrCNE), was introduced in 2007 [11]. In this

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[1] *Abbreviations used:* 2D, two-dimensional; BNE, blue native electrophoresis; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; hrCNE, high-resolution clear native electrophoresis; DDM, dodecyl-β-D-maltoside; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; 1D, one-dimensional; MALDI, matrix-assisted laser desorption/ionization; TOF/TOF, tandem time-of-flight; MS/MS, tandem mass spectrometry; IEF, isoelectric focusing; Opp, oligopeptide permease; ABC, ATP-binding cassette.

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method, the Coomassie dye is substituted with a mixture of an anionic (deoxycholate) and three neutral detergents (dodecyl-β-d-maltoside [DDM], Triton X-100, and digitonin) in cathode buffer. The mixed micelles charged protein complexes and enhanced their migration, resulting in a high resolution under native conditions.

In this study, we optimized BNE and hrCNE for *S. coelicolor* membrane proteins and then characterized the protein complexes by using a combination of native/native and native/SDS 2D gel electrophoresis.

Materials and methods

Bacterial strains and cultural conditions

S. coelicolor M145 was used in the study [2]. Strain maintenance and culture were carried out as described previously [12]. Briefly, spores were pre-germinated. They were then inoculated into 50 ml of Tryptone Soya Broth medium supplemented with 5 g/L yeast extract (TSBY) and 340 g/L sucrose in 250-ml flasks. Cultures were cultivated at 220 rpm and 30 °C for 72 h and were harvested by 6000*g* centrifugation for 10 min. The cell pellet was stored at –70 °C until use.

Preparation of membrane proteins

All procedures were performed at 4 °C unless indicated otherwise. *S. coelicolor* membrane fractions were obtained using a previously described protocol with some modifications [13]. Approximately 5 g of cell paste (wet weight) was resuspended in 25 ml of buffer A (25 mM Tris [pH 7.8], 1 mM ethylenediaminetetraacetic acid [EDTA], and 1 mM phenylmethanesulfonyl fluoride [PMSF]) and broken by passing through an FA-032 French Press (Thermal Electronics) three times at 1.2×10^8 kPa. Cell debris was removed by centrifugation at 15,000*g* for 20 min. The supernatant was then subjected to ultracentrifugation at 190,000*g* for 2 h. The pellet (membrane fractions) was stored at −70 °C until use.

For the extraction of membrane proteins, approximately 0.25 g of membrane fractions was resuspended using 5 ml of buffer B (25 mM Tris [pH 7.8], 1 mM EDTA, and 500 mM 6-aminohexanoic acid) and was sonicated briefly. DDM (from 10% stock solution) was added to a final concentration of 2% (DDM/protein ratio [g/g] ~2.4:1). The sample was solubilized for 30 min and then ultracentrifuged at 190,000*g* for 1 h. The supernatant was collected, and the protein concentration was adjusted to 6 mg/ml. Protein concentration was determined using BCA (bisinchoninic acid) assay (Sigma).

BNE and hrCNE

The BNE was performed as described previously [6,14]. Gradient gels of 8 to 16% were used (gel dimensions: 140 × 160 × 1.5 mm). Anode buffer contains 25 mM imidazole/HCl (pH 7.0). Cathode buffer contains 50 mM Tricine, 7.5 mM imidazole, and 0.02% Coomassie Brilliant Blue G-250.

The hrCNE was carried out as described by Wittig and coworkers [11]. Anode buffer contains 25 mM imidazole/HCl (pH 7.0). For hrCNE–DDM, cathode buffer contains 50 mM Tricine, 7.5 mM imidazole, 0.05% (w/v) sodium deoxycholate, and 0.02% (w/v) DDM. For hrCNE–Triton, 0.05 (v/v) Triton X-100 was used instead of 0.02% (w/v) DDM. In addition, 0.01% (w/v) Ponceau Red was added to samples to mark the running front during electrophoresis.

Conditions for electrophoresis were all the same. For the analysis, 80 μg of membrane proteins was loaded to each lane. The gels were run at 4 °C. The initial voltage was 100 V. When the sample entered the gel, the voltage was adjusted to 500 V and the current was set to 15 mA. BNE and hrCNE gels were stained using Coomassie blue R-250 (Sigma). High molecular weight markers (HMW Native Markers, GE Healthcare) were used to evaluate molecular weight of the protein complexes.

hrCNE/hrCNE 2D electrophoresis

Gradient gels of 8 to 16% were used for the first- and second-dimension gel electrophoresis. Individual lanes of 0.5 cm were excised from one-dimensional (1D) native gels. Gel strips were dipped into water for 2 s. Then the strips were placed on a glass plate at the position usually occupied by the stacking gels. After applying spacers and placing the second glass plate, the glass plates were brought to the upright position. The gradient gel mixture was poured between the glass plates. After full polymerization, the strip was pushed onto the second-dimensional gel and the gaps were filled with a 4% native gel mixture. For hrCNE–DDM/hrCNE–Triton 2D electrophoresis, DDM was used in the cathode buffer for the first dimension and Triton X-100 for the second dimension. For hrCNE–Triton/hrCNE–DDM 2D electrophoresis, Triton X-100 was used in the cathode buffer for the first dimension and DDM for the second dimension. Colloidal Coomassie staining was used according to the standard protocol (Invitrogen).

Native/SDS–PAGE 2D electrophoresis

The native gel lanes of 0.5 cm were excised. The gel strips were boiled for 8 min in SDS buffer containing 2% SDS and 250 mM Tris–HCl (pH 6.8). The strips were assembled between the 2D SDS–PAGE glass plates at the position usually occupied by the stacking gel, and the glass plates were brought to the upright position. The 12% separating gel mixture was poured between the glass plates. Following polymerization, the strip was pushed onto the second-dimensional gel and the gaps were filled with a 4% stacking gel mixture. The 2D gel was stained using the Colloidal Coomassie Kit (Invitrogen). Protein complexes in the 1D map and proteins in the 2D map were analyzed using Quantity One software (Bio-Rad).

In-gel protein digestion and protein identification by mass spectrometry

Coomassie-stained protein spots were excised and sliced into pieces. Gel pieces were destained using 30% acetonitrile in 100 mM NH_4HCO_3. Destained gel pieces were dehydrated using 100% acetonitrile. After shrinking, acetonitrile was removed and gel pieces were dried in a vacuum. Gel pieces were rehydrated in 10 ng/μl trypsin (Promega) in 50 mM NH_4HCO_3 and incubated at 37 °C overnight. The supernatant was removed, and 100 μl of solution (60% acetonitrile and 0.1% trifluoroacetic acid) was added to gel pieces and incubated for 15 min. The supernatants were pooled and dried in a vacuum. Finally, 2 μl of 20% acetonitrile was added to resuspend the peptides.

For mass spectrometry, 1 μl of peptide sample was mixed with 0.5 μl of α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% acetonitrile and 0.1% trifluoroacetic acid and then spotted onto the matrix-assisted laser desorption/ionization (MALDI) target and air-dried. Peptide samples were analyzed on a 4800 MALDI TOF/TOF (tandem time-of-flight) Proteomics Analyzer (Applied Biosystems) equipped with a 355-nm wavelength, an Nd:YAG laser, and a 2-kV acceleration voltage. The mass spectrometry was performed in the positive ion mode, and data were acquired in automatic mode. The mass range was set to 800 to 4000 Da. A second run of tandem mass spectrometry (MS/MS) focused on the eight most intensive peaks of the first mass spectrometry performed. The laser was set to fire 2500 times per spot in MS/MS mode, and collision energy was 2 kV. Mass spectra were carried