Cosynthesis and different expression proteins analysis of antibiotic synthesized-blocked mutants of *Streptomyces roseoflavus* Men-myco-93-63

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Abstract

Streptomyces roseoflavus Men-myco-93-63 was isolated from potato scab (*S. scabies*) decline soil as an antagonistic strain, which can effectively inhibit several phytopathogenic fungi and control important related plant diseases. In this study, cosynthesis and different expression protein analysis between seven antibiotic synthesized-blocked mutants of Men-myco-93-63 were carried out. Two-dimensional gel electrophoresis was used to compare proteins of cosynthesis and non-cosynthesis mutants. By matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, two different protein spots were isolated and identified to be homologous proteins of ectoine hydroxylase and transcriptional regulator (DeoR family protein), which could be related to the biosynthesis of antibiotics of *S. roseoflavus* Men-myco-93-63.

Introduction

Proteome originated from the combination of the words protein and genome. It was proposed by Wilkin and Willams,[1] and refers to a set of proteins used to express a type of gene. Proteome technology and methods have been widely applied in many fields, such as growth regulation and immediate response of cells, regulation of protein phosphorylation and glycosylation, the study of protein function, molecular marker for disease diagnosis, and drug development. As the core technique of proteomic research, over recent years two-dimensional gel electrophoresis (2-DE) has been developed.[2] Through this technology, protein samples from two cells or tissues can be separated under identical conditions, allowing different protein spots to be expressed and compared between two gels. Different protein spots can then be further analyzed by assistive technology.

S. roseoflavus Men-myco-93-63 isolated from soil harboring potato scab (*S. scabies*) disease shows potential application in biological control fields.[3] This strain and its fermentation can effectively inhibit several phytopathogenic fungi and control related plant diseases,[4] such as cotton verticillium wilt *(Verticillium dahliae* Kleb),[5,6] potato scab (*S. scabies*), tomato gray mold *(Botrytis cinerea*), and cucumber powdery mildew (*Sphaerotheca fuliginea*).[7] The antibiotic substances produced by the strain have been purified, and the active components and antagonistic mechanisms are the subject of ongoing research in our laboratory. Obtaining antibiotic synthesized-blocked mutants and developing methods of testing their cosynthesis is one of the best ways to study the synthesis and metabolic pathway of antibiotics. It can provide useful information about changes in synthesis related proteins and metabolic pathways of antibiotics. For example, the biosynthetic intermediates of rifamycin were found by cosynthesis of inactive mutants (non-rifamycin-producing) of *Nocardia mediterranei*.[8-10] By studying the cosynthesis of non-producing mutants of *S. erythreus*, Zhang *et al.*[11] found the possible biosynthesis pathway of erythromycin. Li *et al.*[12] clarified the relationship of blocked sites of mutants and the relative positions of all intermediates in the biosynthetic pathway of apramycin, and proposed a biosynthetic pathway of apramycin according to the results of cosynthesis of blocked mutants.

In this study, a cosynthesis experiment was developed by combining antibiotic synthesized-blocked mutants of Men-myco-93-63, 2-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization - time-of-flight - mass spectrometry (MALDI-TOF-MS) analysis in order to compare different proteins expressed by cosynthesis and non-cosynthesis mutants were carried out. These will provide the theoretical basis for clarification of the biosynthetic pathway of antibiotics produced by *S. roseoflavus* Men-myco-93-63.

Materials and Methods

Strains and culture medium

The wild strain and seven blocked mutants of Men-myco-93-63 were stored in 20% glycerine at -20°C. MV11 MV18 MV21, MV22 and MV35 are antibiotic synthesized-blocked mutants obtained via microwave irradiation. The other two antibiotic synthesized-blocked mutants, DE01and DE02, are DES blocked mutants. V40 (*V. dahliae*) was the indicator strain and the main pathogen of cotton verticillium wilt in the Hebei province of China and this was stored on PDA medium at 4°C. Mycelia of V40 were cultured on TSB medium which was used to extract proteins.

Cosynthesis experiment between every two blocked mutants

Seven blocked mutants were cultured on potato dextrose agar (PDA) medium for five days (5 d), and the spores were diluted with aseptic water to identical concentrations. Every two mutants were combined and 3 μL of two mutant suspension was added to the 6 mm sterile filter paper on the medium at intervals of 0.5 cm, and cultured at 28°C for five days.

Sample preparation

Sample preparation procedures were, as previously described,[13] and protein concentrate was measured by means of standard curve method.

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Two-dimensional gel electrophoresis

2-DE was performed via use of the IPG-phorisoelectric focusing system (Bio-Rad) according to the manufacturer's instructions. Dry gel strips were rehydrated actively for 4 h, followed by passive rehydration for 8 h at 20°C with a mixture containing 8 M urea, 4% (wt/vol) CHAPS, 0.2% (wt/vol) dithiothreitol, and a trace amount of bromophenol blue. For the first dimension, the protein samples were separated on 17 cm long rehydrated Immobiline DryStrips with a non-linear gradient from pH 4 to 7 (Bio-Rad) and were focused at 250V for 30 min, 1000 V for 1 h, 10,000 V for 5 h, and 60,000 V for 1 h. After isoelectric focusing, the IPG strips were reduced, alkylated, and exchanged with detergent. 2-DE was carried out in sodium dodecyl sulfate-12% polyacrylamide gels (29 by 19 cm), and the proteins were visualized by Coomassie Brilliant Blue staining. Images were captured by ScanMarker 8700, and analyzed by computer-assisted gel analysis software PDQust 7.3.1 (Bio-Rad). More than three separate gels were analyzed for each sample. Protein spots which displayed dominant and consistent patterns were selected for further identification.

Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry analysis

The protein spots of interest were excised from the 2-DE gels and mass spectrometry analysis was carried out using the Beijing Genomics Institute Proteomics Platform. The results were analyzed by Mascot software (*http://www.matrixscience.com*).

Results

Cosynthesis between every two blocked mutants

In this step, every two blocked mutants were cosynthesized. Two complementary pairs of mutants (DE02-MV21 and DE02-MV35) were found, while inhibition zones were near MV21 and MV35 (Figure 1). This showed that DE02 was a secretor while MV21 and MV35 were transformants. This indicates that DE02 provided some intermediate metabolites which could be transformed into active substances by MV21 and MV35, while other mutants, such as DE01, MV11, MV18, and MV22, were not.

Analysis of different proteins expressed between cosynthesis mutants (MV21 and MV35) and non-cosynthesis mutants (DE01, MV11, MV18, and MV22)

Different expressed proteins of cosynthesis

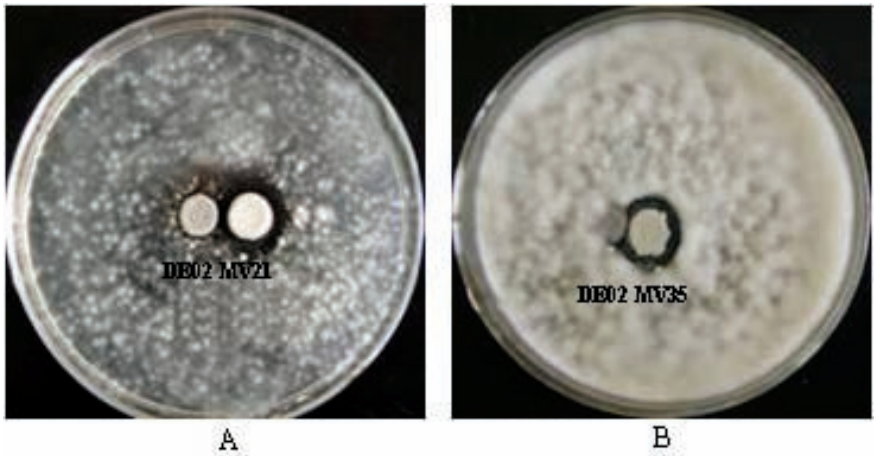

Figure 1. Cosynthesis experiment between blocked mutants of Men-myco-93-63. A) DE02-MV21; B) DE02-MV35.

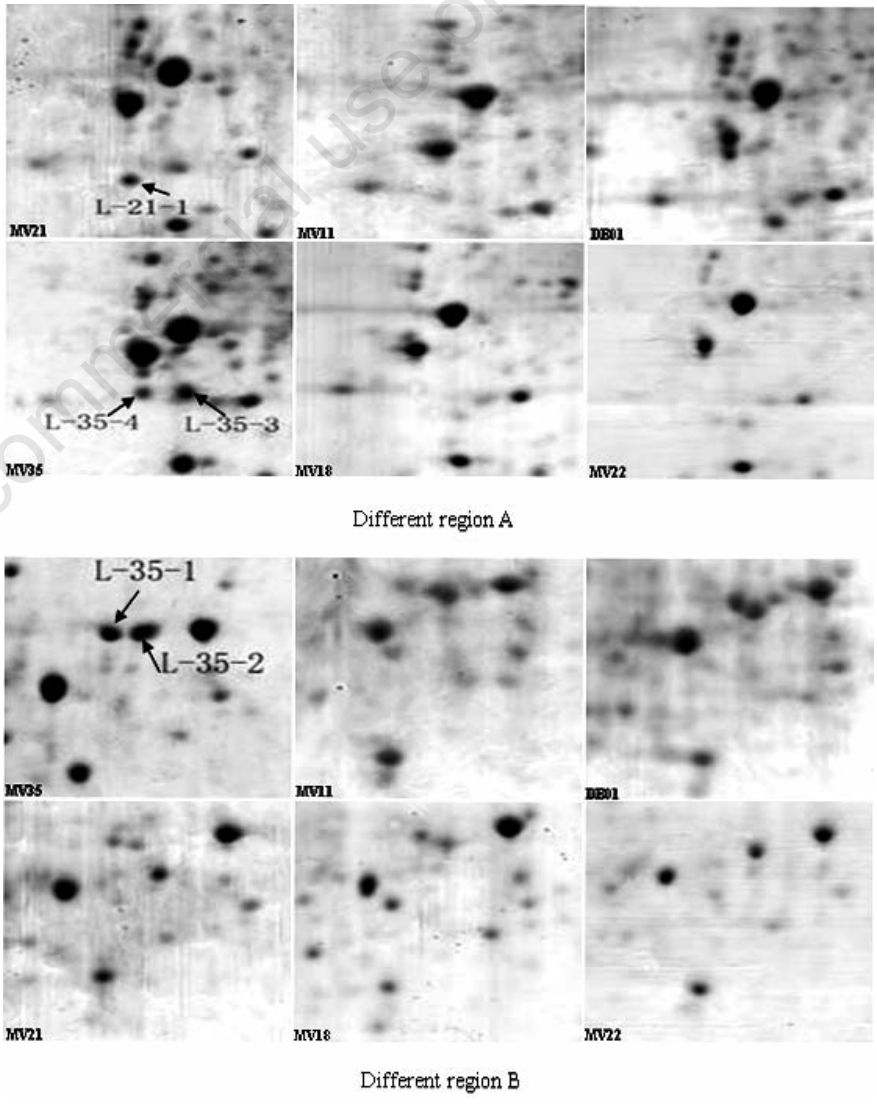

Figure 2. Two different regions of 2-DE images of cosynthesis and non-cosynthesis mutants.

Table1. Peptide mass fingerprinting identification of protein spots L-35-1 and L-35-3.

Spot No.	Theoretical Mr/pI	Protein score	Sequence coverage (%)	Accession No.	Protein name
L-35-1	32.782/5.52	97	43	gil71159311	Ectoine hydroxylase
L-35-3	29.356/5.62	85	46	gil91223394	Transcriptional regulator

mutants (MV21 and MV35), and non-cosynthesis mutants (DE01, MV11, MV18, and MV22) were analyzed by 2-DE. By means of PDQust 7.3.1 software and craft analysis, five different expressed proteins were found. These were stably expressed in mutants MV21 and MV35, but not expressed in DE01, MV11, MV18, and MV22. Different protein regions were amplified (Figure 2A and B).

Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry analysis of different protein spots

Five spots were blasted in the *Streptomyces* protein database with Mascot software (Matrixscience). Two spots, L-35-1 and L-35-3, could be evaluated. Both spots belonged to mutant MV35; one was homologous to ectoine hydroxylase, the other to a transcriptional regulator (Table 1).

Discussion and Conclusions

Research into cosynthesis is one of the best ways to study the biosynthetic pathways of antibiotics produced by a microorganism.[4,8-10,12] In this way, we can identify the relationship between mutation sites of different mutants to confirm which mutant is a secretor and which is a transformant.[14]

In this study, DE02 secreted metabolites while MV35 transformed them into active substances. This illustrates that mutation sites in the strain DE02 are reversed compared to those in MV35 during antibiotic producing processes. Cosynthesis between DE02-MV21 and DE02-MV35 were successful while others were not. Therefore, there may be some exudates produced by MV21 and MV35 which can transform metabolites of DE02 into active antifungal substances. Other mutants are neither secretor nor transformant strains. This means that perhaps some regulatory gene, promoter, or operon in their genome structure has been destroyed, suggesting that some structural genes essential for synthesis of antibiotics become silent, or some related enzymes cannot be translated, thus leading to metabolic pathways blockage.

Expressed proteins of MV21, MV35 with DE01, MV11, MV18, and MV22 were compared by 2-DE. Five different protein spots were found which were stably expressed in mutants of MV21 and MV35, but not in DE01, MV11, MV18, and MV22. Only two spots, L-35-1 and L-35-3, were successfully identified with ectoine hydroxylase and transcriptional regulator (DeoR family protein), respectively. Ectoine hydroxylase of *S. anulatus* is an important compatible solute in moderately halophilic bacteria,[15,16] which can keep intra and extracellular turgor pressure balanced when microorganisms are in a hypertonic environment.[17,18] Ectoine has a higher solubility and non-ionic property under physiological pH which makes it the most important osmo-regulator in halophilic photosynthetic bacteria.[19] Prabhu *et al.*[20] has reported that the formation of hydroxyectoine in the industrial ectoine producer *Halomonas elongata* was improved by the heterologous expression of the ectoine hydroxylase gene, *thpD*, from *S. chrysomallus.* The efficient conversion of ectoine to hydroxyectoine was achieved by the concerted regulation of *thpD* by the *H. elongata ectA* promoter. In this study, the L-35-1 spot may help biocontrol strains adjust osmotic pressure to ensure its normal metabolic progress. The protein could be expressed in MV35, but not in the wild strain or DE02, so it may be regulated negatively by some active and antibacterial substance. The reason it was expressed only in MV35 may be that MV35 was unable to secrete precursor substances that control its transformation into antibacterial substances.

Upon analysis, the L-35-3 protein was found to be homologous with a transcriptional regulator of the DeoR protein family. This indicates that during cosynthesis experiments the L-35-3 protein might have combined with the promoter or operon of the synthetic gene for the transformation substance secreted by MV35, and helped the transformation substance convert the precursor substance into active material. This active substance then formed the inhibition zone around MV35. The exact function of this protein, however, needs to be the subject of further study.

In addition to protein spots L-35-1 and L-35-3, we found other spots with different expression levels. It was, however, difficult to identify them because of their instability during electrophoresis, and the limitations of 2-DE, identification techniques and the Streptomyces database. Many different proteins were expressed during this study that may have been related to antibiotic synthesis but could not be successfully matched to their homologous proteins in the database. Their identification and relationship to the synthesis of antibiotics still needs to be clarified by additional experiments.

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