Time Dependent Change in the DNA-Binding Proteins of *Streptomyces coelicolor* A3(2)

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(Received Dec. 3, 1990 / Accepted Feb. 1, 1991)

Streptomyces coelicolor A3(2) is a typical member of the *Streptomyces* which undergoes complex morphological development and produces a series of secondary metabolic products during stationary phase. It is the best understood and most highly studied member of this group of organisms¹. *Streptomyces* shown temporal expression of a number of morphological and secondary metabolic functions, the control of which should reside in a series of DNA-protein interactions at the genome level. Some of the DNA binding-proteins which are involved in these interactions should also show time dependent expression. This study is aimed at the identification of such DNA binding proteins.

A number of methods of isolation of DNA binding-proteins have been described: the use of DNA-cellulose column²; the extraction of chromatin³; and the electrophoretic separation of proteins followed by probing with radiolabelled DNA⁴. The third method was chosen because it is simple and rapid, allowing for the processing of a large number of samples and because it is a highly sensitive method of detection of a wide range of DNA-binding proteins. It can also be used to detect sequence specific DNA-binding proteins.

Detection of DNA binding proteins

The *S. coelicolor* A3(2) strain M130 obtained from Professor D.A. Hopwood, John Innes Institute, Norwich, UK was used throughout this study. All cultures were grown at 30°C. Sample of cells used for the preparation of cell free extracts were grown with vigorous shaking in 100 ml of MJPP medium; 50mM KH₂PO₄, 15mM (NH₄)₂SO₄, 0.8mM MgSO₄, 0.07mM CaCl₂, 1% casamino acids, 0.2% yeast extract, 0.2% glycerol. Inoculation of 100 µl of a 1.5 x 10⁷ colony forming unit spore suspension took place at a set time each day in order to allow

harvesting of a series of cultures of different ages on the same day. Growth and antibiotic production of the cultures were monitored by isolating the mycellium by centrifugation in a preweighed centrifuge tube at 15,000 rpm in a Sorval SS-34 rotor for 10 minutes at 4°C. After the wet weight of the pellet was recorded it was resuspended in 5ml of 0.1 M NaOH and shaken gently at 30°C for 15 minutes. Each sample was then centrifuged at 10,000 rpm in a Sorval SS-34 rotor for 10 minutes at 4°C. Actinorhodin concentration in the supernatant was measured by spectrophotometric analysis by scanning and measuring optical density at 535 nm on a Beckman DV 8 Spectrophotometer.

Chromosomal DNA was prepared by Method 2 as described by Hopwood *et al.*⁵ and purified by caesium chloride gradient centrifugation. The total chromosomal DNA was labelled with (P³²)dCTP using a nick translation kit from Amersham Plc, UK. Single stranded DNA was obtained by heating 50 µl of labeled DNA to 100°C for 10 minutes and then adding 950 µl of ice cold blocking buffer followed by storage on ice until needed.

Cell free extracts of the cultures were obtained by spinning down the mycellium at 7,000 rpm in a Sorvall GSA rotor for 10 minutes at 4°C. The pellet was washed in 50 ml of distilled water and spun down in a preweighed centrifuge tube at 15,000 rpm in a Sorvall SS-34 rotor for 10 minutes at 4°C. The wet weight of cells was recorded. The sample was resuspended in 10 ml of Buffer 1 (20 mM Tris-HCl, 10mM MgCl₂, 2mM CaCl₂, 1mM EDTA, 0.5mM Dithiothreitol, pH 8.1) and the sample passed through a Yeda press five times. The lysate was treated with 20 µg ml⁻¹ DNAase 1 for 90 minutes at 15°C. The cell debris was removed by centrifuging the lysate at 15,000 rpm in a Sorvall SS-34 rotor for 10 minutes at 4°C. EDTA was added to a final concentration of 20 mM. Finally, the lysate was freeze dried and resuspended at 400 µl of Buffer 2 (20mM Tris-HCl, 50mM NaCl, 5mM EDTA, 1mM mercaptoethanol, pH 8.1) per 1 g of original wet weight; the samples were then dialysed against Buffer 2 for 24 hours with three changes of buffer. The protein concentration was determined by the method of Bradford⁶ and the samples stored at -20°C.

The proteins were separated by discontinuous SDS-PAGE electrophoresis^{7,8)} and the proteins transferred to nitrocellulose by electroblotting for 15-18 hours at 0.2-0.4 A⁹⁾. After transfer, the membrane was blocked for 3 hours at 30°C with Blocking buffer (1mM EDTA, 10mM Tris-HCl, 2% Bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.05 M NaCl, pH 7.0). The membrane was then transferred to fresh Blocking buffer containing P³² labelled DNA and incubated at 30°C for 24 hours. After probing, the membrane was washed in Washing buffer (1mM EDTA, 10mM Tris-HCl, 2% Bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.05M-0.06M NaCl, pH 7.0) for 3 hours with three changes of buffer. The membrane was then dried and autoradiographed at -70°C with Fuji enhancer screens. In all cases, the molecular weights of the DNA-binding proteins were calculated from S³⁵ labelled molecular weight markers.

Time course of DNA binding protein expression

In Figure 1, Lane C we can see the characteristic profile of the DNA-binding proteins from a three day old culture *S. coelicolor*. Fourteen or more proteins can be detected with molecular weights ranging from 13kD to 130kD. Two of these of molecular weight 25kD and 30kD were not detected when single stranded DNA was used as a probe (data not

shown) and thus must be dsDNA specific DNA-binding proteins. The remainder bind both ssDNA and dsDNA; no specific ssDNA-binding proteins were detected.

The changes in the profiles of DNA-binding proteins over eight days are also shown in Figure 1. Dramatic changes in the DNA-binding protein profiles can be seen with specific induction of proteins from Day 1 to Day 3 followed by specific switch off of proteins from Day 4 to Day 6 such that a very simplified profile remains from Day 6.

Four broad classes of DNA-binding proteins can be identified according to changes in their expression over time. Group 1 proteins (including the 19 kD, 19.5 kD and 21 kD proteins) are continuously expressed over the whole eight days with some quantitative changes. Group 2 proteins (including the 13-16 kD, 30 kD and 35 kD proteins) are present on Day 1 but are completely switched off by Day 6. Group 3 (including the 18.6 kD, 25 kD and 26 kD proteins) proteins are switched on between Day 1 and Day 5 and then switched off by Day 6, while those of Group 4 (including the 32.2 kD, 80 kD and 130 kD proteins) show profiles varying from the above criteria. These groups are by their nature heterogeneous, especially Group 3 in which maximum expression can vary from Day 2 to Day 5, allowing subclassification. In general, however, the ability to classify the DNA-binding proteins in this way suggests that there is coordinated control of expression of these genes responsible for their production. It should be noted, however, that the DNA-binding protein profile is likely to vary depending upon the media and growth conditions used.

Three different approaches were used to try and identify sequence-specific DNA-binding proteins in *S. coelicolor*: probing with a clone of the *bldA* locus¹⁰⁾; probing with clones of the *act* gene cluster¹¹⁾ and a comparison of the binding to total chromosomal DNA from *S. coelicolor* and herring sperm DNA. No sequence-specific binding to *bldA* or the *act* cluster was detected (data not shown). This is not unexpected in the case of *bldA* as the gene has been identified as coding for a rare leucine tRNA¹²⁾ and control of expression may be via specific sigma factors binding to RNA polymerase¹³⁾.

In order to determine which of the DNA-binding

Fig. 1. Changes in DNA-binding protein profile of *Streptomyces coelicolor* A3(2) over 8 days. A: day 1; B: day 2; C: day 3; D: day 4; E: day 5; F: day 6; G: day 7; H: day 8. Washing was carried out using 0.05 M NaCl.

Fig. 2. Detection of DNA binding proteins of *Streptomyces coelicolor* A3(2) which are sequence specific. The autoradiographs show one day (top) and three day (bottom) exposures. DNA probes and washing buffer (NaCl) concentrations (M) were as follows.

Lane	A	B	C	D	E	F	G	H
DNA probe	← Herring Sperm →				← Streptomyces →			
Washing buffer	0.05	0.15	0.20	0.30	0.30	0.20	0.15	0.05

proteins were sequence specific the proteins were probed with DNA that is likely to show little homology with *Streptomyces* DNA. Herring sperm DNA was chosen as the non-homologous probe. It was considered that the binding characteristics of proteins that bind non-specifically would remain unchanged, while sequence-specific binding proteins would different features depending on the target DNA. The DNA-binding proteins from *S. coelicolor* fell into three classes when probed with labeled *S. coelicolor* or herring sperm DNA and compared (Figure 2).

Class I, exemplified by the M_r 130 kD, 80 kD, 58 kD, 50 kD and 44.5 kD proteins showed similar binding profiles with both DNAs at all salt concentrations. These are probably non-specific binding proteins. The Class II proteins, M_r 30 kD and 25 kD, which were previously identified as dsDNA specific DNA-binding proteins, remained bound to *Streptomyces* DNA at higher salt concentrations compared to Herring DNA; although the binding to *Streptomyces* DNA dropped between 0.05M and 0.15M NaCl, it then stabilised in contrast to the binding to Herring DNA which continued to de-

crease. These are probably sequence-specific DNA-binding proteins which recognise defined sequences in the *S. coelicolor* genome. The initial loss of binding as the salt concentration is increased could reflect the loss of DNA that is bound non-specifically, while sequence-specific binding is stronger and maintained at higher salt concentrations. Class III proteins, M_r 35 kD and 26 kD, show the reverse pattern when binding to Herring DNA is compared to that with *Streptomyces* DNA. The 35 kD protein suddenly decreased in binding to *Streptomyces* DNA at 0.15M NaCl compared to a progressive loss of binding for herring sperm DNA. The 26 kD protein showed decreased binding to *Streptomyces* DNA at 0.05M NaCl compared to a progressive loss of binding with Herring DNA. There are two possible explanations for the results with these proteins: firstly that the recognition site for these sequence-specific DNA-binding proteins is present in high numbers per unit length in the herring genome (for example in the repeated DNA sequences), compared to a low number per chromosome in *S. coelicolor*; secondly, the length of the binding site could be quite short, thus the number of pseudosites in Herring DNA would be large due to its length relative to the *Streptomyces* genome and because pseudosites in the *Streptomyces* would be selected out. The progressive loss of binding with Herring DNA compared to a specific cut off for *Streptomyces* supports this as the pseudosites would be expected to have a variety of binding specificities.

Importance of DNA binding proteins

This work has identified a large number of DNA-binding proteins from *S. coelicolor*. A subgroup of these proteins which are dsDNA specific and/or sequence specific are important. These show a definite cut off of expression during the secondary metabolic phase of growth of the organism, all being shut off by Day 6. These proteins show a divergence of levels of expression and times of maximum expression. The importance of the DNA-binding proteins to the control of temporally controlled genes of *S. coelicolor* should not be underestimated.

SUMMARY

A series of DNA-binding proteins from *Strepto-*

myces coelicolor A3(2) were identified and shown to change in cellular level throughout the growth cycle of the organism. Certain of these DNA-binding proteins were shown to be specific for sequences found in the *Streptomyces coelicolor* A3(2) genome.

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