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A novel protein that binds to *dnrN–dnrO* intergenic region of *Streptomyces peucetius* purified by DNA affinity capture has dihydrolipoamide dehydrogenase activity

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ABSTRACT

An antitumour chemotherapeutic, daunorubicin (DNR), produced by *Streptomyces peucetius* exhibits cytotoxic activity through topoisomerase-mediated interaction with DNA, thereby inhibiting DNA replication and repair and RNA and protein synthesis. It is synthesized by the type II polyketide pathway. Understanding molecular mechanisms that drive expression of antibiotic biosynthetic genes in response to diverse signals and chemical inducers is of considerable interest. Intergenic DNA between regulatory genes *dnrN* and *dnrO* of DNR biosynthesis pathway in *S. peucetius* has a promoter for transcription of *dnrN* in one strand and three promoters in the opposite strand for *dnrO*. Studies have shown that DnrO binds to a specific sequence in this region to activate transcription of *dnrN*. In the present study, using biotinylated intergenic DNA in combination with streptavidin magnetic beads, we have purified a protein that binds to this target sequence. The protein has been characterized by nano LC ESI MS/MS mass spectrometry. Sequence similarity searches for effective identification of protein by genome databases comparisons led to identification of a sequence-specific DNA binding protein that exhibits dihydrolipoamide dehydrogenase (DLDH) activity suggesting that this protein may be involved in regulation of DNR biosynthesis.

Introduction

Streptomyces are soil bacteria that undergo intricate growth pattern consisting of morphological and physiological differentiation that coincides with the production of a plethora of secondary metabolites including antibiotics [1,2]. These developmental processes are controlled at different points by regulatory genes, some of which have been identified and functionally characterized. However, a comprehensive picture of regulatory network for antibiotic biosynthesis has not emerged [3,4]. Understanding molecular mechanisms that drive expression of antibiotic biosynthetic genes in response to diverse signals and chemical inducers is of considerable interest. In *Streptomyces*, along with many other regulators, transcription is controlled by a family of proteins known as *Streptomyces* Antibiotic Regulatory Proteins (SARP)[1] that exert their effect on gene expression by binding to DNA sequences within promoters [5]. Some of the antibiotic regulatory genes in *Streptomyces* include, *bldD* in *S. coelicolor* [6,7], *strR* in *S. gresius* [8], *farA* in *S. lavendulae* [9], *lndI* in *S. globisporous* [10], *actII orf IV* in *S. avermitilis* [11] and *novG* in *S. spheroids* [12].

Daunorubicin (DNR) is an antitumour chemotherapeutic agent that is synthesized by the type II polyketide pathway. Regulation of DNR biosynthesis is by three genes *dnrO* [13], *dnrN* [14] and *dnrI* [15]. Mutation affecting function of any one of the three regulatory genes leads to cessation of DNR biosynthesis. DNR biosynthesis commences by the activation of *dnrO* transcription. DnrO apparently binds to its cognate sequence (37 bp sequence that overlaps with *dnrO*'s P1 promoter) within the 433 bp intergenic region between *dnrN* and *dnrO* genes to activate *dnrN* [16] (Fig. 1). DnrN is a pseudo-response regulatory protein that binds upstream to the un-translated region of *dnrI* gene – the key regulator, and activates it [14]. DnrI binds to several upstream elements of structural genes to activate DNR biosynthesis [15]. Feed back inhibition of DNR biosynthesis has been demonstrated in *Streptomyces peucetius* by the formation of drug–protein complex between DNR and DnrO [16]. DNR–DnrO complex fails to bind at the cognate sequence and thereby *dnrN* is not activated. The *dnrN–dnrO* intergenic region comprises of three active promoters for *dnrO* and one promoter for *dnrN* (Fig. 1). The 5′ un-translated region of *dnrN* overlaps with the transcripts from OP2 and OP3 promoters (Fig. 1) of *dnrO* [13]. Presence of multiple promoters for *dnrO*, divergent overlapping transcripts of *dnrN–dnrO* and DNA intercalating properties of DNR were factors that prompted us to investigate the possibility of a higher order regulatory network.

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[1] *Abbreviations used:* DNR, daunorubicin; DLDH, dihydrolipoamide dehydrogenase; SARP, *Streptomyces* Antibiotic Regulatory Proteins; NDM, Nitrate Defined Medium; PEI, polyethyleneimine; PMF, peptide mass fingerprint.

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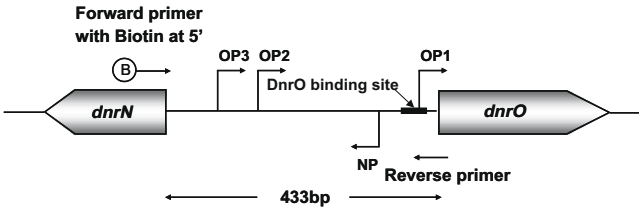

Fig. 1. Schematic representation of the two divergent genes *dnrN* and *dnrO* separated by 433 bp intergenic region. Position of promoters and the primer binding sites are shown. OP represents promoter for *dnrO* and NP is *dnrN* promoter.

Affinity chromatography is a well established technique for purifying transcription factors due to its selective affinity for a specific DNA sequence [17]. Many purification methods have been standardized for isolation of DNA binding proteins of high purity [18–20]. Current genetic techniques such as one hybrid assay [21] and phage display [22] lack the ability to detect accessory proteins that help in sequence specific binding. Several pull down strategies have been developed using the prototype of Gabrielson et al. [23] that utilize biotinylated DNA immobilized to streptavidin coated magnetic beads. This method minimizes the use of multiple rounds of purification in columns and also yields DNA binding protein in its active form. Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry is a powerful technique which uses mass-to-charge ratio (m/z) of peptides resulting in a peptide mass fingerprint which enables identification from databases [24]. MALDI-TOF, however is not efficient enough to identify proteins from un-sequenced genomes [25]. With the arrival of liquid chromatography nano spray tandem mass spectrometry (nano LC MS/MS); analysis of full peptide sequences [26] is now possible. MS/MS data can be used for sequence similarity searches for effective identification (cross species identification) of proteins from un-sequenced genomes [27]. Sequence similarity search engines like MS BLAST [27] and FASTS [28] have been successfully used in identification of many proteins. Transcription factors usually bind to their cognate DNA sequence with affinities in the picomolar range and have a 10^3–10^5 higher affinity for this specific sequence than for any other DNA sequences [17]. This high binding affinity is utilized for the purification of DNA binding proteins. This report describes purification of a protein that binds to *dnrN–dnrO* intergenic region by magnetic DNA affinity capture; which was identified as dihydrolipoamide dehydrogenase (DLDH) by mass spectrometry.

Materials and methods

Reagents and chemicals

Streptavidin paramagnetic beads (Promega, Madsion, USA) were used for capturing biotinylated DNA. Biotin labeled forward primer was synthesized (Sigma Aldrich Chemicals Pvt. Ltd) for amplification of intergenic DNA. *Taq* polymerase (Invitrogen Paisley, UK) was used for amplification of intergenic DNA. Ni-Agarose (Sigma Aldrich Chemicals Pvt. Ltd) was used for affinity purification His-tagged protein. Bacterial culture media components were obtained from standard commercial manufacturer (HiMedia Laboratories Pvt. Ltd., India).

Bacterial strains and plasmids

S. peucetius 29050 was obtained from American Type Culture Collection, Rockville, MD, USA. Plasmid clone of *dnrN* and *dnrO* (pdnrNO) was a gift from Prof. C.R. Hutchinson (University of Wisconsin, Madison). Bluescript SK- (Stratagene) plasmid DNA was used as binding competitor in the affinity capture strategy to minimize non-specific binding of proteins in the cell lysate. Expression vector pQE31 (Qiagen, Valencia, CA, USA) and *Escherichia coli* M15pREP4 host were used for protein expression.

Amplification of the Intergenic region

Biotinylated forward primer, OIntF (5′-Biotin-GCTCTTCAGCAAT CACGAC) and reverse primer, OIntR (5′-GTCCCTCTCCAGAACAG) were utilized to amplify 551 bp DNA that comprises of 433 bp intergenic region, 35 bp and 43 bp of N-terminal part of *dnrN* and *dnrO* genes, respectively. Plasmid vector pUC18 carrying *dnrN* and *dnrO* genes (pdnrNO) were used as template in the amplification. Standard DNA amplification parameters were followed [29].

Immobilization of biotinylated DNA to magnetic beads

Streptavidin coated magnetic beads were washed three times for 10 min each in TE buffer. Fifteen microgram of biotinylated DNA in 200 μl of TE buffer was mixed with 1.5 mg of streptavidin coated magnetic beads and incubated at 30 °C in a rotating wheel. After 2 min of incubation, magnetic field was applied and 1 μl of clear supernatant was removed and set aside for loading agarose gel electrophoresis. The process was repeated and second sample was removed after 2 min. A total of ten samples were drawn at 2 min intervals. These samples were loaded on agarose gel electrophoresis to estimate the efficiency of biotinylated DNA binding to streptavidin beads. The immobilized DNA bound to the beads was stored in TE buffer (pH 7.0) at 4 °C for further use.

Extraction of DNA binding proteins from S. peucetius

Cellular proteins were extracted from 10 g wet weight of 48 h *S. peucetius* culture grown in Nitrate Defined Medium (NDM) at 30 °C. Cell pellet was suspended in 20 mM Tris (pH 7.5) containing 10 mM $MgCl_2$ and 7 mM β-mercaptoethanol. After two washes in the buffer, cells were disrupted by sonication at 38% amplitude in Vibracell sonicator and centrifuged at 12,000 rpm (RPR20-2 rotor, Hitachi) for 15 min at 4 °C. To the supernatant, polyethyleneimine (PEI) was added slowly with constant and gentle stirring at 4 °C to a final concentration of 1%. Incubation was continued for 1 h with constant stirring. PEI precipitated proteins were collected by centrifuging the mixture at 10,000 rpm (RPR20-2 rotor, Hitachi) for 10 min. The protein pellet was extracted with 200 mM NaCl in 20 mM Tris (pH 7.5). This was repeated twice and the pooled extract was precipitated with 80% ammonium sulfate. The sample was finally stored at −20 °C in binding buffer containing 20 mM Tris pH 8.0, 1 mM EDTA, 50 mM KCl, 1 mM DTT and 15% glycerol.

Affinity purification of DNA binding proteins

Affinity purification was performed as described by Gabrielson et al. [23] with minor modifications. Two mg of enriched DNA binding proteins in 0.5 ml of binding buffer was used for affinity capture of sequence-specific DNA binding proteins. Addition of competitor DNA reduced the contamination of non-specific DNA binding proteins as described earlier [30]. Enriched protein was first incubated with 6 μg of pBluescript SK^- competitor plasmid DNA for 1 h at 30 °C. Subsequently magnetic beads containing immobilized DNA was added and incubated for 1 h at 30 °C with continuous gentle stirring. Immobilized DNA was separated from the mixture in the presence of magnetic field. Immobilized DNA with bound proteins was washed for 5 min duration three times with 150 mM KCl in 20 mM Tris pH 8.0, 1 mM EDTA and 15% Glycerol. DNA bound proteins were repeatedly eluted three times with 1 M KCl in 20 mM Tris pH 8.0, 1 mM EDTA and 15% Glycerol. Eluted