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Research paper

Isolation and molecular characterization of a stationary phase promoter useful for gene expression in *Gordonia*

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ABSTRACT

Gordonia are gram-positive bacteria belonging to Actinomycetes family with a wide variety of industrial and environmental applications. The genetic toolbox, however, is limited for manipulation of these organisms. In the present study, a new promoter has been isolated from *Gordonia* sp. IITR 100 and characterized in detail. The promoter was found to be functional in *Escherichia coli*. The minimal promoter was identified in a 166 bp fragment by deletion mapping. The putative -35 and -10 hexamer showed four and five nucleotide matches respectively with the *E. coli* consensus sequence. Three direct repeats and an imperfect inverted repeat upstream to -35 were found. The isolated promoter was found to be six times stronger than the P*kan* promoter observed by cloning *lacZ* downstream to each of them in a plasmid in *E. coli*. The β-galactosidase activity was maximum at stationary phase and found to be ~800 MU for *Gordonia* sp. IITR 100 and *E. coli*. This is the first report of a stationary phase promoter isolated and characterized from *Gordonia*.

1. Introduction

Promoters are one of the key sequences that regulate gene expression. They can be classified either based on the structure or expression. Structurally promoters can be grouped into two types, one which contains the conserved hexameric sequences at -10 and -35 region (Youderian et al., 1982; Harley and Reynolds, 1987), and the other which contains an extended -10 region including a 5′-TGN-3′ motif in addition to the -10 region (Keilty and Rosenberg, 1987). Most of the promoters from gram-positive bacteria are reported to have an extended -10 region (Sabelnikov et al., 1995; Agarwal and Tyagi, 2003). Based upon the expression, promoters can be either constitutive or regulated (inducible or repressible). Constitutive promoters express throughout the growth whereas inducible or repressible promoters are regulated based upon growth phase, stress factors or some external factors. Regulated promoters are desirable for production of proteins as the growth phase and the protein production phase can be uncoupled (Delic et al., 2013). Such promoters have been reported from several bacteria such as *Escherichia coli* (Aldea et al., 1990; Miksch and Dobrowolski, 1995; Kang et al., 1997), *Lactococcus lactis* (Madsen et al., 1999), *Bacillus* (Lee et al., 2010) etc.

Actinomycetes have recently attracted attention because of their tremendous applications in industrial and environmental biotechnology. Molecular tools have been developed for various members of *Rhodococcus*, *Streptomyces* and *Corynebacteria*. Several inducible promoters, which are induced by methanol (Kagawa et al., 2012) or ε-caprolactam (Komeda et al., 1996) have been reported from *Rhodococcus rhodochrous* J1. Similarly, iron regulated promoter P*desA* from *Streptomyces pilosus* (Flores et al., 2003), thiostrepton inducible promoter P*tipA* from *Streptomyces lividans* 66 (Murakami et al., 1989) have also been reported. A number of promoters viz. P*dapA*, P*dapB*, P*ilvA*, P*ilvC* and P*ofmP* have been characterized from *Corynebacterium glutamicum* (Patek et al., 1996).

Amongst Actinomycetes, the genus *Gordonia* has drawn much interest in recent years because they can be used for the transformation (Shavandi et al., 2009; Kampfer et al., 2013), synthesis (Arenskotter et al., 2004) and biodegradation of several types of chemical compounds (Linos et al., 1999; Lo Piccolo et al., 2011; Chanthamalee and Luepromchai, 2012; Lin et al., 2012; Indest et al., 2013). However, these applications are rarely realized because of the limited genetic tools available to manipulate these organisms. A few cloning vectors for *Gordonia* have been reported which are based on the pRC4 replicon

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from *Rhodococcus* (Arenskotter et al., 2003). P*dsz* is the only promoter experimentally characterized, which is responsible for the expression of biodesulfurization enzymes from *Gordonia alkanivorans* (Shavandi et al., 2010).

The present study involves isolation of a novel stationary phase promoter from *Gordonia* sp. IITR100 that functions in *E. coli* also.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* and *Gordonia* sp. were cultivated in Luria broth at 30 °C. Antibiotics kanamycin (50 μg/ml) and tetracycline (12 μg/ml) were used wherever necessary.

2.2. Genomic DNA isolation from Gordonia

Genomic DNA was isolated from *Gordonia* sp. IITR100 following the protocol by Marmur (1961) which was modified as follows. Briefly, single colony was inoculated in 50 ml luria broth and allowed to grow for three days to reach an OD_{600} ~ 1.2. The culture was harvested at 12,000 rpm for 1 min followed by washing with 1 ml Milli Q water. The pellet was resuspended in 12 ml of solution I containing 10 mM Tris-Cl pH 8.0, 1 mM EDTA and 0.35 M sucrose. Lysozyme (50 mg) and 120 μl RNase A (10 mg/ml) were added to it and incubated at 37 °C for 1 h. This was followed by addition of 225 μl proteinase K (10 mg/ml) and incubation was done at 55 °C for 6 h with gentle inversion of the tubes in between. Twelve milliliters of SDS (2% stock solution) was added and incubation was continued at 55 °C for 2 h. DNA was obtained after phenol: chloroform (1:1) extraction and ethanol precipitation, and dissolved in 5 ml TE buffer (100 mM Tris-Cl, 10 mM EDTA, pH 8.0).

2.3. Recombinant DNA techniques

Plasmid from *E. coli* was isolated using genelute plasmid mini kit (SIGMA, USA) following manufacturer's instructions. Plasmid isolation and electroporation in *Gordonia* was done following a protocol optimized in our laboratory (Singh and Srivastava, 2013). Restriction enzyme digestion, ligation and transformation were performed using standard methods (Sambrook et al., 1989). Restriction enzymes, ligase and phosphatase were obtained from NEB, USA.

2.4. Construction of promoter library

In order to isolate promoters from *Gordonia*, genomic DNA was digested with restriction enzyme RsaI and fragments of about 500–1000 bp were gel eluted. Promoter probe vector pMC1871 (Pharmacia, Piscataway, NJ), which can replicate in *E. coli*, was digested with SmaI followed by dephosphorylation using Antarctic phosphatase. Different ratios of vector and insert were ligated and used to transform chemically competent *E. coli* DH5α cells. Clones were screened on X-gal containing plates. The control plasmid pPOS11 was constructed by ligating the PstI digested promoterless *lacZ* fragment from plasmid pMC1871 with PstI digested linearized plasmid pRSG43 (Fig. 1).

2.5. Deletion mapping of the promoter

The PCR primers used for deletion mapping of promoter region are shown in Table S1 and Fig. 2. Primer pairs PS 8 & PS 15R2, PS 9 & PS 15R2, PS 10 & PS 15R, PS 11 & PS 15R, PS 12 & PS 15R, PS13 & PS 15R, PS12 & PS16R, PS12 & PS25R were used for amplification by PCR using phusion polymerase (NEB, USA) and pPOS1 as template. The PCR products obtained by primer pairs PS 8 & PS15R2 and PS 9 & PS15R2 were digested with StuI (since there was a site for restriction enzyme SmaI inside the 731 bp promoter-containing fragment) and ligated to SmaI digested plasmid pMC1871 (Fig. 2). The remaining PCR products were digested with SmaI and ligated with SmaI digested plasmid pMC1871. A series of plasmids namely pPOS2-10 were constructed (Fig. 3). The *E. coli* transformants were selected on X-gal containing plates. The blue colonies were selected and β-galactosidase assay was done. For determination of activity in *Gordonia*, the small fragment containing promoter and *lacZ* was isolated from plasmids pPOS2-pPOS10 by digestion with PstI, gel eluted and ligated to PstI digested plasmid

Table 1
Bacterial strains and plasmids used in the present study.

Bacterial strain or plasmids	Relevant description	Reference
Escherichia coli DH5α	Strain for cloning	Invitrogen
Gordonia sp. IITR100	Biodesulfurizing bacterium Gen bank accession number GU084407	(Singh and Srivastava, 2013) MCC 2877
Plasmids		
pMC1871	*E. coli* promoter probe vector, 7476 bp, Tet^R	Pharmacia, Piscataway, NJ
pGP1-2	*E. coli* plasmid expresses T7 RNA polymerase, used as the source of Pkan promoter in the present study, 7200 bp, Kan^R	(Tabor and Richardson, 1985)
pRSG43	*E. coli-Rhodococcus* (*Gordonia*) shuttle vector, 5241 bp, Kan^R	(Shavandi et al., 2009)
pPOS1	pMC1871 containing 731 bp fragment, Tet^R	This study
pSC1	pRSG43 having PstI fragment containing *lacZ* along with 731 bp promoter from pPOS1, Kan^R	This study
pPOS11	pRSG43 having PstI fragment containing *lacZ* without promoter from pMC1871, Kan^R	This study
pPOS2	pMC1871 containing 632 bp promoter fragment, Tet^R	This study
pPOS3	pMC1871 containing 532 bp promoter fragment, Tet^R	This study
pPOS4	pMC1871 containing 428 bp promoter fragment, Tet^R	This study
pPOS5	pMC1871 containing 332 bp promoter fragment, Tet^R	This study
pPOS6	pMC1871 containing 232 bp promoter fragment, Tet^R	This study
pPOS7	pMC1871 containing 184 bp promoter fragment, Tet^R	This study
pPOS8	pMC1871 containing 132 bp promoter fragment, Tet^R	This study
pPOS9	pMC1871 containing 197 bp promoter fragment, Tet^R	This study
pPOS10	pMC1871 containing 215 bp promoter fragment, Tet^R	This study
pPOS11	pRSG43 having PstI fragment containing promoter less *lacZ*, from pMC1871, Kan^R	This study
pPOS12	pRSG43 having PstI fragment containing *lacZ* along with 632 bp promoter from pPOS2, Kan^R	This study
pPOS13	pRSG43 having PstI fragment containing *lacZ* along with 532 bp promoter from pPOS3, Kan^R	This study
pPOS14	pRSG43 having PstI fragment containing *lacZ* along with 431 bp promoter from pPOS4, Kan^R	This study
pPOS15	pRSG43 having PstI fragment containing *lacZ* along with 332 bp promoter from pPOS5, Kan^R	This study
pPOS16	pRSG43 having PstI fragment containing *lacZ* along with 232 bp promoter from pPOS6, Kan^R	This study
pPOS17	pRSG43 having PstI fragment containing *lacZ* along with 184 bp promoter from pPOS7, Kan^R	This study
pPOS18	pRSG43 having PstI fragment containing *lacZ* along with 132 bp promoter from pPOS8, Kan^R	This study
pPOS19	pRSG43 having PstI fragment containing *lacZ* along with 197 bp promoter from pPOS8, Kan^R	This study
pPOS20	pRSG43 having PstI fragment containing *lacZ* along with 215 bp promoter from pPOS8, Kan^R	This study
pPOS21	pMC1871 containing P*kan* promoter, Tet^R	This study
pPOS22	pRSG43 having PstI fragment containing *lacZ* along with P*kan* promoter from pPOS9, Kan^R	This study