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Transcription of the *rpsO-pnp* operon of *Streptomyces coelicolor* involves four temporally regulated, stress responsive promoters

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ABSTRACT

Primer extension with RNA from an RNase III null mutant of *Streptomyces coelicolor* M145 and a primer complementary to the polynucleotide phosphorylase gene revealed two major extension products. Two different extension products were observed using RNA from either wild type M145 or the null mutant with a primer complementary to *rpsO*. Mapping of the 5′-ends of these extension products to the *rpsO-pnp* intergenic region indicated that all four putative transcription start sites were preceded by possible promoter sequences. These putative promoters were synthesized by the PCR and cloned into pIPP2, a *xylE*-based streptomycete promoter probe vector. Transfer of the pIPP2 derivatives to *S. coelicolor* and catechol dioxygenase assays demonstrated that all four cloned fragments had promoter activity in vivo. The activities of the four promoters changed over the course of growth of *S. coelicolor* and studies in three sigma factor mutant strains demonstrated that three of the promoters were σ^B dependent. Northern blotting studies showed that the levels of the *rpsO-pnp* transcripts remained relatively constant over the course of growth of *S. coelicolor* M145, but that on a molar basis, the levels of the readthrough and *pnp* transcripts were considerably lower than those of *rpsO*. PNPase is a cold shock protein in *S. coelicolor* and the activity of the *rpsO-pnp* promoters increased during cold shock at 10°, resulting in a two-fold increase in PNPase activity, compared with the activity at 30°.

1. Introduction

The *rpsO-pnp* operon, encoding ribosomal protein S15 (*rpsO*) and polynucleotide phosphorylase (*pnp*) is conserved in many bacterial species (Clarke and Dowds, 1994; Luttinger et al., 1996; Régnier and Portier, 1986). In *Escherichia coli*, the operon is transcribed from two promoters, one upstream of *rpsO* and one in the intergenic region between *rpsO* and *pnp* (Portier and Regnier, 1984; Régnier and Portier, 1986). Transcription produces three messenger RNAs, an *rpsO* transcript, produced via the termination of transcription at a rho-independent terminator at the end of the gene, a readthrough transcript, initiated upstream of *rpsO* but terminated downstream of *pnp*, and a *pnp* transcript, initiated at the intergenic promoter and terminated at the same site as the readthrough transcript (Portier and Regnier, 1984; Régnier and Portier, 1986). Additionally, expression of the *rpsO-pnp* operon is regulated by RNase III cleavage of an intergenic hairpin, situated between *rpsO* and *pnp* (Régnier and Portier, 1986). This cleavage is the basis for the autoregulation of polynucleotide phosphorylase (PNPase) expression in *E. coli* (Jarrige et al., 2001; Robert-Le Meur and Portier, 1992, 1994). In the most recent model proposed for this process in *E. coli*, PNPase digests the 5′-fragment produced by RNase III cleavage at the intergenic hairpin and the residual *pnp* mRNAs are degraded in an RNase E-dependent pathway (Carzaniga et al., 2009).

We have recently studied expression of the *rpsO-pnp* operon in the soil-dwelling actinomycete, *Streptomyces coelicolor*. The organization of the operon is conserved in that organism (see further below) and we have shown that RNase III cleavage of an intergenic hairpin is involved in autoregulation of PNPase expression, as is the case in *E. coli* (Gatewood et al., 2011). In an earlier study, an intergenic promoter was identified in a sister organism, *Streptomyces antibioticus*, by cloning the *rpsO* intergenic region in a high copy number promoter probe vector (Bralley and Jones, 2004). To date, however, we have not analyzed the *rpsO-pnp* promoters in *S. coelicolor*.

We report here the detailed analysis of the *rpsO-pnp* promoters in *S. coelicolor*. To our surprise, we discovered that the genes of the operon are transcribed from four promoters, two upstream of *rpsO* and two in

Abbreviations: APE, acetone-phosphate-EDTA; $CATO_2$ase, catechol dioxygenase; PCR, polymerase chain reaction; PNPase, polynucleotide phosphorylase; SFM, soya flour-mannitol; SMM, *Streptomyces* minimal medium.

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the *rpsO-pnp* intergenic region. These promoters are of different strengths and are temporally regulated. Moreover, the activity of each promoter increases during cold shock of *S. coelicolor* and these changes in activity result in a ca. two-fold increase in PNPase activity levels as compared with the activity in mycelium incubated at 30 °C. These findings are considered in the overall context of the regulation of *rpsO-pnp* expression in *S. coelicolor*.

2. Materials and methods

2.1. Growth of organisms

S. coelicolor M145, J1668, J1501 and their derivatives were grown on SFM agar to produce spores and in SMM liquid medium with carboxymethylcellulose as the dispersant, as previously described (Gatewood and Jones, 2010; Gatewood et al., 2011; Kieser et al., 2000). For some experiments mutant strains were grown on R2YE or R5 media (Kieser et al., 2000). *E. coli* strains XL-1 Blue (Stratagene, La Jolla, CA) and ET12567/pUZ8002 (Gust et al., 2003) were grown on L-broth containing antibiotics as necessary. When necessary apramycin was used at a final concentration of 50 μg/ml. Conjugation mixtures were plated on SFM agar. Growth of the various *S. coelicolor* strains in liquid medium was measured as optical density at 450 nm as described previously (Bralley and Jones, 2001).

2.2. Primer extension and cloning of putative promoters

RNA was isolated from mid-log phase cultures of *S. coelicolor* M145 and from its *rnc* mutant, JSE1880, as described previously (Chang et al., 2005; Gatewood et al., 2011). Primer extensions were performed as described previously (Chang et al., 2005) using primers +15R to identify putative transcription start sites for *rpsO* and +16R to identify the putative sites for *pnp*. The sequences of these two primers are shown in Fig. 3.

PCR products were prepared from regions suspected to contain *rpsO-pnp* promoters using the primer sets P*rpsO*A and P*rpsO*B for *rpsO* promoters and P*pnp*A and P*pnp*B for the promoters thought to be situated in the intergenic region between *rpsO* and *pnp*. The sequences covered by these primers are shown in Fig. 3 and the forward and reverse primers are designated as F and R, respectively, in the figure. The 5′-primers P*rpsO*AF, P*rpsO*BF, P*pnp*AF and P*pnp*BF, contained *Xba*I sites while the 3′-primers P*rpsO*AR, P*pnp*AR and P*pnp*BR contained *Bam*HI sites. As the fragment of interest corresponding to P*rpsO*B contained a *Bam*HI site, P*rpsO*BR contained an *Xba*I site. Thus, the fragments representing P*rpsO*A, P*pnp*A and P*pnp*B were cloned as *Xba*I/*Bam*HI fragments, while the fragment corresponding to P*rpsO*B was cloned as an *Xba*I fragment. The template used for PCR was pJSE602, which is a pBluescript SK+ derivative containing the entire *rpsO-pnp* operon flanked by 224 additional bp at the 5′-end and 81 bp at the 3′-end (Gatewood et al., 2011). Conditions for PCR were as described previously (Chang et al., 2005).

PCR fragments were digested with *Xba*I and *Bam*HI (P*rpsO*A, P*pnp*A, P*pnp*B) or with *Xba*I (P*rpsO*B) and were cloned into the integrative, *xylE*-based promoter probe vector, pIPP2, previously developed in this laboratory (Jones, 2011). Ligation mixtures were used to transform *E. coli* XL-1 Blue and the desired plasmid constructs were identified by restriction digestion and by the PCR. The cloned inserts were 117 bp (P*rpsO*A), 348 bp (P*rpsO*B), 343 bp (P*pnp*A) and 349 bp (P*pnp*B) in size. Plasmids corresponding to each promoter were used to transform *E. coli* ET12567/pUZ8002 and the resulting promoter probe constructs were then transferred to *S. coelicolor* M145 by conjugation from the *E. coli* strains. pIPP2 alone was transferred to *S. coelicolor* M145 and J1501 via conjugation to provide control strains. The presence of the desired constructs in the *S. coelicolor* derivatives was confirmed by the PCR.

S. coelicolor strains containing null mutations in the *hrdA*, *hrdC* and *hrdD* genes were generously provided by Dr. Mark Buttner, along with the parental strain, J1668. Strains containing mutations in *sigB*, *sigH* and *sigL* were generously provided by Dr. Jung-Hye Roe, along with J1501. In all cases, spores of these strains were conjugated with the relevant *E. coli* strains containing the *rpsO-pnp* promoter constructs. The promoter probe constructs and pIPP2 alone were also transferred to the parental strains, J1668 (*hrd* mutants) and J1501 (*sig* mutants).

2.3. Measurement of promoter activity

Mycelium was grown in liquid SMM medium as shown in Fig. 4 and samples of 15–25 ml were removed at various times after inoculation. Mycelium was washed with APE buffer and extracts were prepared by sonication (Branson 250 Sonifier, 30–45 s, output = 4) in 1–2 ml of APE buffer (Sala-Trepat and Evans, 1971a, 1971b) and protein was determined using the BioRad dye binding reagent with bovine serum albumin as a standard. The in vitro assay for catechol dioxygenase was performed as described previously (Hsieh and Jones, 1995; Jones, 2011) with results expressed as milliunits of activity/min/mg protein. Control experiments were performed to ensure that the amounts of extract used were in the linear region of the enzyme vs. activity curve.

For the cold shock experiments, mycelium was grown in 250 ml of SMM medium for 13 h at 30 °C. Samples were removed at this point for catechol dioxygenase and PNPase assays and stored at −80 °C. Half of each culture was then transferred to a fresh flask and incubated for 3 h at 10 °C. Samples were removed from the 10 °C and 30 °C cultures at 0.5, 1, 2 and 3 h following the transfer to 10 °C. Mycelial samples for PNPase assay were washed with 0.9% NaCl and sonicated in a buffer composed of 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 5% glycerol and 1 mM dithiothreitol. PNPase activity was measured as described previously with [^{3}H]ADP as the substrate for polymerization. In Fig. 6, PNPase activities are expressed relative to the value measured at 5 h post-inoculation, set arbitrarily to 100.

2.4. Northern blotting

Total RNA was isolated from 5, 15 and 25 h *S. coelicolor* cultures, grown on SMM medium, as previously described (Chang et al., 2005; Gatewood et al., 2011). Northern blotting was performed as previously described (Gatewood et al., 2011) after fractionation of 5 or 10 μg portions of each RNA preparation on 5% polyacrylamide gels containing 7 M urea. RNAs were transferred to Brightstar-Plus membranes (Ambion) by electroblotting and membranes were hybridized to DNA probes. *rpsO* and *pnp* specific probes were prepared as described previously (Gatewood et al., 2011). Radioactive probes were prepared by random priming and hybridization and washing were carried out at 42° following the instructions provided in the Ambion NorthernMax-Gly kit. The intensity of the *rpsO*, readthrough and *pnp* transcript bands was measured by densitometry. The band densities were then

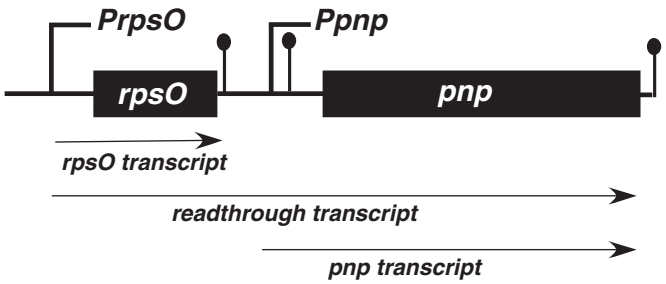

Fig. 1. Schematic representation of the bacterial *rpsO-pnp* operon. P*rpsO* and P*pnp* represent the upstream and intergenic promoters found in *E. coli* and certain other bacteria. The ball-and-stick structures immediately following *rpsO* and *pnp* represent rho-independent transcription terminators. The ball-and-stick structure just upstream of *pnp* represents the intergenic hairpin which is cleaved by RNase III. The diagram is not drawn to scale.