Gene 538 (2014) 280–287

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

The σ^F-specific anti-sigma factor RsfA is one of the protein kinases that phosphorylates the pleiotropic anti-anti-sigma factor BldG in *Streptomyces coelicolor* A3(2)

Erik Mingyar, Beatrica Sevcikova, Bronislava Rezuchova, Dagmar Homerova, Renata Novakova, Jan Kormanec *

Institute of Molecular Biology, Slovak Academy of Sciences, 845 51 Bratislava, Slovak Republic

ARTICLE INFO

Article history:
Accepted 10 January 2014
Available online 23 January 2014

Keywords:
Anti-sigma factor
Promoter
Partner-switching mechanism
Phosphorylation
S1-nuclease mapping
Sigma factor

ABSTRACT

The anti-anti-sigma factor BldG has a role in the morphological differentiation and antibiotic production of *Streptomyces coelicolor* A3(2). Together with the anti-sigma factor UshX it is involved in the "partner-switching"-like activation of the sigma factor σ^H that has a dual role in the osmotic stress response and morphological differentiation in *S. coelicolor* A3(2). Although BldG is phosphorylated in vivo in *S. coelicolor*, neither of the interacting anti-sigma factors UshX and ApgA is found to phosphorylate it. By using a combination of several approaches, we demonstrated a direct interaction between BldG and the anti-sigma factor RsfA, which has been previously shown to regulate antibiotic production and morphological differentiation in *S. coelicolor* and to specifically interact with the sporulation-specific sigma factor σ^F. RsfA phosphorylates BldG in vitro, demonstrating that RsfA is a specific kinase for BldG and negatively regulates its activity. However, another interacting anti-anti-sigma factor homolog, SCO0869, was not phosphorylated by RsfA. Transcriptional analyses of *rsfA* revealed a single promoter, the activity of which was repressed by osmotic stress and decreased during differentiation. These data suggested that BldG has a pleiotropic role in the regulation of at least two sigma factors, σ^H and σ^F, in *S. coelicolor*.

1. Introduction

The Gram-positive soil-dwelling bacteria of the genus *Streptomyces* are characterized by their ability to produce a wide variety of secondary metabolites. They undergo a complex process of morphological differentiation involving aerial hyphae erected on mycelial colonies, which finally undergo septation into chains of unigenomic spores that are essential for the dispersal of these bacteria (Chater, 2000). In their natural habitat, they are exposed to a variety of nutritional and environmental stresses, and regulation of morphological differentiation has been found to be coupled with stress-related signals (Vohradsky et al., 2000). The ability to modulate gene expression in response to these stresses is mediated in many bacteria in part by alternative RNA polymerase sigma factors, which direct the expression of genes encoding stress proteins essential for overcoming these unfavorable conditions. The general stress–response sigma factor σ^B of *Bacillus subtilis* is the best-characterized example in Gram-positive bacteria; its activity is regulated by an RsbW/RsbV partner-switching mechanism. Under non-stress conditions, σ^B is sequestered by the anti-sigma factor RsbW. Release of the sigma factor from this complex is accomplished by the anti-anti-sigma factor RsbV, which is dephosphorylated by specific PP2C-type phosphatases under stress conditions and sequesters RsbW. In addition, RsbW specifically phosphorylates RsbV through the serine protein kinase activity of its HATPase_c domain, thereby providing negative feedback on σ^B activation by inactivating RsbV (Hecker et al., 2007; Price, 2002).

The genome of the most completely studied model streptomycete, *Streptomyces coelicolor* A3(2), contains 65 genes encoding sigma factors, including 9 close homologs of *B. subtilis* σ^B. This heterogeneity of sigma factors shows that regulation at the transcriptional level is quite complex (Bentley et al., 2002; Hahn et al., 2003). Characterization of these nine σ^B homologs revealed roles in the control of morphological differentiation and in the osmotic stress response. For instance, σ^F, σ^N, and σ^K are involved in the control of morphological differentiation (Dalton et al., 2007; Mao et al., 2009; Potuckova et al., 1995) while σ^I appears to have a role in the osmotic stress response (Homerova et al., 2012). Interestingly, σ^H and σ^B have been suggested to regulate both morphological differentiation and the osmotic stress response (Kelemen et al., 2001; Kormanec et al., 2000; Lee et al., 2005; Sevcikova et al., 2001; Viollier et al., 2003). These results indicate that there is a connection between stress responses and differentiation in *S. coelicolor* A3(2). The regulation of these σ^B homologs in *S. coelicolor* seems to be more complex than that of σ^B in *B. subtilis*, since the former contains genes for as many as 45 homologs of the *B. subtilis* RsbW and 15 homologs of

Abbreviations: BACTH, bacterial two-hybrid system; bp, base pair(s); BSA, bovine serum albumin; CFU, colony-forming units; DTT, dithiothreitol; *E, Escherichia*; LB, Luria–Bertani (medium); IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; *S, Streptomyces*; SDS, sodium dodecyl sulfate; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid; TSP, transcription start point(s); WT, wild-type.

* Corresponding author. Tel.: +421 2 59307419; fax: +421 2 59307416.
E-mail address: jan.kormanec@savba.sk (J. Kormanec).

0378-1119/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2014.01.041

RsbV (Mittenhuber, 2002). A partner-switching-like mechanism for sigma factor activation has also been described in *S. coelicolor* A3(2). An anti-sigma factor RsbA with an anti-anti-sigma factor RsbV is involved in the osmotic stress activation of σ^B (Lee et al., 2004). Moreover, σ^I is regulated by the anti-sigma factor PrsI and anti-anti-sigma factor ArsI (Homerova et al., 2012). Another anti-sigma factor, RsfA, has been found to negatively regulate antibiotic production and morphological differentiation in *S. coelicolor* and to interact with the sporulation-specific σ^F and with SCO0781 and SCO0869, two anti-anti-sigma factor homologs (Kim et al., 2008). An anti-anti-sigma factor BldG has been shown to be involved in the osmotic stress activation of σ^H by sequestering its anti-sigma factor UshX/PrsH (Sevcikova et al., 2010). This regulation seems to be even more complex since BldG also interacts with anti-sigma factor ApgA, though no target sigma factor has yet been identified for ApgA (Parashar et al., 2009; Sevcikova et al., 2010). The *bldG* gene has been shown to have a pleiotropic function, controlling both antibiotic production and differentiation in *S. coelicolor* A3(2) (Bignell et al., 2000; Champness, 1988). BldG contains a highly conserved serine residue (Ser57) which is presumably phosphorylated by the serine kinase activity of BldG's cognate anti-anti sigma factors. This serine has been shown to be reversibly phosphorylated in *S. coelicolor*, and this phosphorylation is essential for the regulation of antibiotic production and morphological differentiation (Bignell et al., 2003). In the present study, we identify and characterize a candidate anti-sigma factor involved in this phosphorylation. Employing several approaches, RsfA, which was previously characterized and suggested to be an antagonistic regulator of σ^F (Kim et al., 2008), is shown to specifically interact with BldG and phosphorylate it. In addition, we investigated the expression of the *rsfA* gene during morphological differentiation of *S. coelicolor* A3(2) and after exposure to osmotic stress.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are described in Table 1. The growth and transformation of *Escherichia coli* were described in Ausubel et al. (1995). The bacteria were grown in LB medium. Growth was monitored by measurement of absorbance at 600 nm (OD_{600}). If required, the medium was supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, 34 μg/ml chloramphenicol, and 100 μg/ml streptomycin. The growth of *S. coelicolor* A3(2) was carried out as described in Kieser et al. (2000). For RNA isolation from surface culture, spores (10^8 CFU) from *S. coelicolor* strains were spread on sterile cellophane membranes placed on minimal medium (MM) in the presence of 0.5% (w/v) mannitol as a carbon source, and grown at 30 °C. Mycelium was harvested at time points which corresponded to the different stages of development (after 1, 2, 3, 4 and 5 days). For RNA isolation from liquid grown cultures, spores (10^9 CFU) from *S. coelicolor* strains were inoculated in 50 ml of liquid NMP medium (Kieser et al., 2000) containing mannitol (0.5% w/v) as a carbon source, and grown at 30 °C to end of exponential phase (20 h), and subjected to the following stress conditions: 30 and 60 min 0.5 M NaCl, and 30 and 60 min 1 M sucrose.

2.2. Bacterial two-hybrid system investigation of protein–protein interactions

BACTH (Karimova et al., 1998) was used to detect interactions between proteins. This system is based on the functional complementation of the *Bordetella pertussis* adenylate cyclase fragments T18 and T25 in a *cya*-deficient *E. coli* strain. Full-length gene constructs in the compatible expression plasmids pUT18C and pKT25 were generated by PCR amplification of the entire genes using chromosomal DNA from *S. coelicolor* M145 as a template and selected primers (Table 2) to introduce an *Xba*I site next to the translation initiation codon and an *Acc*65I site downstream of the stop codon. The amplified DNA fragments were digested with *Xba*I and *Acc*65I, and then ligated into pUT18C or pKT25 plasmids, which had been digested by the same restriction enzymes. To ensure a higher fidelity of DNA synthesis during PCR, *Pfu* DNA polymerase (Stratagene, USA) was used. For the *rsfA* gene, the primers RSFADHDir and RSFADHRev amplified a 440-bp DNA fragment that was cloned in pKT25, resulting in pKT25-rsfA. The *SCO0869* gene was amplified using the primers 0869DHDir and 0869DHRev and cloned as a 380-bp fragment in pUT18C, resulting in pUT18C–SCO0869. The nucleotide sequences of all constructs were checked by sequencing. The plasmids pUT18C-bldG and pKT25-prsI have been described previously (Homerova et al., 2012; Sevcikova et al., 2010). The interaction of fusion proteins encoded in pUT18C-zip and pKT25-zip (Karimova et al., 1998) was used as a positive control.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotypes and relevant characteristics[a]	Reference or source
Strains		
S. coelicolor M145	Wild-type, prototrophic SCP1$^-$ SCP2$^-$ Pgl$^+$	Kieser et al. (2000)
E. coli DH5α	F$^-$ *supE44* Δ*lacU169* (ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Host strain for plasmid cloning and propagation	Invitrogen
E. coli BL21(DE3)pLysS	F$^-$ *ompT hsdS* (r_B^- m_B^-) *dcm*$^+$ *gal met* λ (DE3) pLysS (Cm^R) Host strain for overexpression from pET plasmids	Novagen
E. coli BTH101	F$^-$ *cya99 araD139 galE15 galK16 rpsL1* (Str^R) *hsdR2 mcrA1 mcrB1* Adenylate cyclase deficient host strain for BACTH system	Karimova et al. (2001)
Plasmids		
pET28a	Km^R, expression plasmid with T7 *lac* promoter	Novagen
pET-rsfA	Km^R, pET28a containing *rsfA* under T7 *lac* control	This study
pET-SCO0869	Km^R, pET28a containing *SCO0869* under T7 *lac* control	This study
pET-bldG	Km^R, pET28a containing *bldG* under T7 *lac* control	Sevcikova et al. (2010)
pET-apgA	Km^R, pET28a containing *apgA* under T7 *lac* control	Sevcikova et al. (2010)
pET-prsI	Km^R, pET28a containing *prsI* under T7 *lac* control	Homerova et al. (2012)
pET-ushX1	Km^R, pET28a containing *ushX* under T7 *lac* control	Sevcikova et al. (2010)
pKT25	Km^R, pSU40 derivative containing *B. pertusis* T25 fragment of adenylate cyclase for C-terminal fusions	Karimova et al. (1998)
pUT18C	Ap^R, pUC19 derivative containing *B. pertusis* T18 fragment of adenylate cyclase for C-terminal fusions	Karimova et al. (1998)
pKT25-zip	Km^R, pKT25 containing leucine zipper domain of the yeast GNC4 activator fused to the T25 fragment	Karimova et al. (1998)
pUT18C-zip	Ap^R, pUT18C containing leucine zipper domain of the yeast GNC4 activator fused to the T18 fragment	Karimova et al. (1998)
pKT25-rsfA	Km^R, pKT25 containing the *S. coelicolor rsfA* gene fused to the T25 fragment	This study
pKT25-prsI	Km^R, pKT25 containing the *S. coelicolor prsI* gene fused to the T25 fragment	Homerova et al. (2012)
pUT18C-bldG	Ap^R, pUT18C containing the *S. coelicolor bldG* gene fused to the T18 fragment	Sevcikova et al. (2010)
pUT18C-SCO0869	Ap^R, pUT18C containing the *S. coelicolor SCO0869* gene fused to the T18 fragment	This study

[a] Cm^R, chloramphenicol resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Str^R, streptomycin resistance.