RheA, the repressor of hsp18 in Streptomyces albus G

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In *Streptomyces albus*, Hsp18, a protein belonging to the family of small heat-shock proteins, can be detected only at high temperature. Disruption of *orfY*, located upstream and in the opposite orientation to hsp18, resulted in an elevated level of hsp18 mRNA at low temperature. Genetic and biochemical experiments indicated that the product of *orfY*, now called RheA (Repressor of hsp eighteen), directly represses *hsp18*. In *Escherichia coli*, an hsp18–bgaB transcriptional fusion was repressed in a strain expressing *S. albus* RheA. DNA-binding experiments with crude extracts of *E. coli* overproducing RheA indicated that RheA interacts specifically with the hsp18 promoter. Transcription analysis of rheA in the *S. albus* wild-type and in rheA mutant strains suggested that RheA represses transcription not only of hsp18 but also of rheA itself.

**Keywords**: heat regulation, repressor, RheA, hsp18

**INTRODUCTION**

All organisms respond to heat by increasing the production of a set of proteins, called the heat-shock proteins (HSPs), which play important roles in the adaptive response. The principal function of HSPs is to ensure maintenance of protein integrity by acting on the cellular machinery catalysing protein folding, secretion, repair and degradation. Some HSPs (GroEL, DnaK, smHSPs) are molecular chaperones, which facilitate the folding of cellular proteins (Georgopoulos & Welch, 1993; Jakob et al., 1993). Others, such as the Clp family, are proteases that degrade incorrectly folded proteins.

Although the induction of HSPs is a universal response, organisms have diverse regulatory mechanisms for controlling HSP synthesis. The heat-shock response and its regulation were first studied in *Escherichia coli*, where two heat-shock regulons, positively controlled at the level of transcription by specific sigma factors (σ²⁴ and σ³²), were identified (for reviews see Bukau, 1993; Yura et al., 1993). In *Bacillus subtilis*, at least four classes of heat-inducible genes are distinguished by their regulatory mechanisms (Derré et al., 1999b; Hecker et al., 1996). The thermoregulation of class I genes (*groE, dnaK*) depends on a repressor (HrcA) that interacts with an inverted repeat motif called CIRCE (for controlling inverted repeat of chaperone expression) (Schulz & Schumann, 1996; Yuan & Wong, 1995; Zuber & Schumann, 1994). Class II heat-shock genes are positively regulated by the σ³⁸ factor, the synthesis and activity of which are increased under stress conditions. Class III genes (*clpP, clpC* operon, *clpE*) are negatively regulated by CtsR (class three stress gene repressor), which recognizes a directly repeated heptanucleotide operator sequence (Derré et al., 1999a, b). Class IV comprises heat-shock genes of unknown regulation. Heat-shock regulation mediated by CIRCE and σ³² coexists in some bacteria, for instance *Agrobacterium tumefaciens*, *Caulobacter crescentus* and *Zymomonas mobilis* (Avedissian & Gomes, 1996; Barbosa et al., 1994; Mantis & Winans, 1992; Michel, 1993; Reisenauer et al., 1996; Roberts et al., 1996; Segal & Ron, 1993). In *Bradyrhizobium japonicum*, the heat-shock regulation system consists of mechanisms described previously (Babst et al., 1996) as well as novel systems. Recently, Narberhaus et al. (1998) showed that a conserved DNA element of approximately 100 bp called ROSE (for repression of heat-shock gene expression) confers heat inducibility upon a σ³²-type promoter by serving as a binding site for a putative regulatory protein. The ROSE system regulates genes that encode small HSPs (*hspA–hspH*), the σ³²-like transcription factor (rpoH) and degP (Münchbach et al., 1999).

*Streptomyces* spp. are Gram-positive soil bacteria that undergo morphological differentiation and produce a wide variety of metabolites during growth. Their response to heat shock has been studied mostly in two
species, Streptomyces coelicolor and Streptomyces albus. In S. albus, there are at least three different regulatory networks controlling the synthesis of different HSPs. Unlike in several Gram-positive bacteria, including Bacillus subtilis, dnaK and groEL of Streptomyces are not regulated by the same mechanism: heat regulation of groEL involves a CIRCE motif and an HrcA repressor (Grandvalet et al., 1998), whereas the dnaK operon and clpB are regulated by the HspR repressor, which binds to an inverted repeat called HAIR (HspR-associated inverted repeat) (Bucca et al., 1993, 1997; Grandvalet et al., 1997, 1999). hspR is the last gene of the dnaK operon.

A third heat regulation mechanism, repressing hsp18, has been identified in S. albus. Transcription from a streptomyces vegetative promoter of hsp18, encoding a small HSP, is strongly induced following heat shock (Servant & Mazodier, 1995). An open reading frame (orfY), located 150 bp upstream and in the opposite orientation to hsp18, contributes to the transcriptional regulation of hsp18. Indeed, disruption of orfY generated mutants that synthesized a large amount of hsp18 mRNA at low temperature (Servant & Mazodier, 1996). In addition, heat induction of the hsp18 gene is subject to post-transcriptional regulation, the mechanism of which is unknown. Here we report that the orfY gene, now referred to as rheA (repressor of hsp eighteen), encodes the repressor of hsp18. We also investigated the regulation of rheA in S. albus by in vivo transcriptional analysis. RheA appears to function as a negative autoregulator.

METHODS

Bacterial strains, media, culture conditions and plasmids. S. albus G strain J1074, defective for both Sall restriction and modification systems (Chater & Wilde, 1980), was obtained from the John Innes Culture Collection, and grown in yeast extract/malt extract (YE/M) rich liquid medium (Hopwood et al., 1985). NE medium was used for growth of Streptomyces on plates (Hopwood et al., 1985). Thiostrptomycin was added to a final concentration of 25 µg ml⁻¹. Escherichia coli TG1 (Gibson, 1984) was used as the general cloning host and E. coli BL21/DE3 (Studer & Moffatt, 1986) was used for overproduction of protein by the T7-promoter expression vector. E. coli strains were grown in Luria–Bertani (LB) broth supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (20 µg ml⁻¹) as appropriate. pUC19, pSU2718 and pSU2719 have been described previously (Martinez et al., 1985). pET11a (Novagen) was obtained by introducing the thiostrepton-resistance gene (hirata et al., 1986), encoding a thermostable β-galactosidase, was constructed from pPM1745. This plasmid carries the ampicillin-resistance gene and contains hsp18 and rheA from S. albus (Servant & Mazodier, 1995). A BamHI–SacI fragment of pDL containing bgab was ligated between the BamHI and SacI sites of pPM1745 to give pPM7001. The rheA gene was deleted from this plasmid by SalI digestion to yield pPM7002. pPS310 was digested with Xbal and BamHI. The Xbal site is upstream from and adjacent to the Ndel site. The fragment containing rheA was inserted between the Xbal and BamHI sites of pSU2718 and pSU2719 to give pPM7003 and pPM7004, respectively. In pPM7004, rheA is expressed from the plac promoter, and in pPM7003 rheA is in the opposite orientation. pSU2718 and pSU2719 contain the chloramphenicol acetyltransferase gene (cat) and are based on the pACYC184 replicon (Martin et al., 1988). They are compatible with CoE1-derived plasmids like pMMB702.

Double transformants of E. coli TG1 containing pPM7002 and either pMM7003 or pPM7004 were selected on ampicillin and chloramphenicol. The activity was assayed by measuring the level of β-galactosidase in cells grown to mid-exponential phase at 37°C. The results represent the mean of duplicate assays.

DNA manipulations. Plasmid construction and transformation of E. coli were as described in Sambrook et al. (1989). Plasmid DNA was extracted and purified from E. coli with the Qiagen plasmid kit. Restriction enzymes were used as recommended by the manufacturers. DNA was sequenced by the dye-terminator method (Sanger et al., 1977) with the Sequenase kit (Pharmacia).

Plasmid construction. To overproduce RheA in E. coli, rheA (ATG to TGA) was inserted into the expression vector pET11a. A corresponding PCR fragment amplified from S. albus genomic DNA with oligonucleotides PS90 (5'-GGTGATCCCTAGGACGCCCGGAC-3') and PS92 (5'- TTGATCATACTGACCAGCCCGCCGCGCC-3') was digested with Ndel and BamHI and inserted into pET11a cut with Ndel/BamHI to obtain pPS310.

A DNA fragment corresponding to rheA and upstream sequence containing the hsp18 promoter was isolated from S. albus genomic DNA by PCR using oligonucleotides PS90 (5’-GGTGATCCCTAGGACGCCCGGAC-3’) and PS97 (5’-GGTGATCCAGAATCAGGACCTTTAGTG-3’). The fragment was digested with BamHI and inserted into the BamHI site of pUC19 to give pPS311.

A plasmid containing a transcriptional fusion between the hsp18 promoter and the Bac. steareothermophilus bgab gene (Hirata et al., 1986), encoding a thermostable β-galactosidase, was constructed from pPM1745. This plasmid carries the ampicillin-resistance gene and contains hsp18 and rheA from S. albus (Servant & Mazodier, 1995). A BamHI–SacI fragment of pDL containing bgab was ligated between the BamHI and SacI sites of pPM1745 to give pPM7001. The rheA gene was deleted from this plasmid by SalI digestion to yield pPM7002. pPS310 was digested with Xbal and BamHI. The Xbal site is upstream from and adjacent to the Ndel site. The fragment containing rheA was inserted between the Xbal and BamHI sites of pSU2718 and pSU2719 to give pPM7003 and pPM7004, respectively. In pPM7004, rheA is expressed from the plac promoter, and in pPM7003 rheA is in the opposite orientation. pSU2718 and pSU2719 contain the chloramphenicol acetyltransferase gene (cat) and are based on the pACYC184 replicon (Martin et al., 1988). They are compatible with CoE1-derived plasmids like pMMB702.

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Overproduction of RheA in \textit{E. coli}, pPS310 and pET11a were introduced into \textit{E. coli} BL21(DE3) in which the T7 RNA polymerase gene is under the control of the inducible lacUV5 promoter. Cultures of strains containing pET11a or pPS310 were induced by adding 1 mM IPTG. Five hours after induction, cells were harvested by centrifugation, resuspended in 50 mM NaCl/B buffer (B buffer: 10 mM Tris, pH 7.5; 10 mM MgCl$_2$; 1 mM DTT; 0.1% Triton X-100; 1 mM EDTA; 10% glycerol) and subjected to sonication. The extract was clarified by centrifugation at 10000 \(g\) for 15 min at 4 \(°C\) and the supernatant was used directly as a crude extract in gel retardation assays.

DNA mobility shift assays. A 300 bp BamHI–SalI fragment from pPS311 containing the \textit{hsp18} and \textit{rheA} promoters was purified and used in gel retardation assays. This fragment was end-labelled with \([\alpha-32\text{P}]\)dATP using the Klenow fragment of DNA polymerase I (Gibco-BRL). The labelled fragment was further purified using the Quiquick PCR purification kit (Qiagen). Proteins were incubated for 10 min at 30 or 37 \(°C\) with the labelled DNA fragment (0-2 pmol) in a 20 \(\mu\)l reaction mixture containing 1 \(\mu\)g calf thymus DNA, 50 mM NaCl, 10 mM Tris (pH 7.5), 1 mM DTT, 10 mM MgCl$_2$, 1 mM CaCl$_2$ and 10% glycerol. Samples were then loaded directly on to a 6% polyacrylamide gel (50 mM Tris, pH 8.5; 400 mM glycine; 173 mM EDTA; 2.5% glycerol) for electrophoresis at 150 V (14 V cm$^{-1}$). The gels were dried and autoradiographed. The DNA fragments used in competition assays were the \textit{hsp18} fragment (specific unlabelled competitor DNA) and the 305 bp \textit{groES} gene of \textit{S. albus} (Servant et al., 1993) (‘non-specific’ competitor DNA).

**RESULTS**

**Regulation of \textit{hsp18} promoter activity in \textit{E. coli}**

To determine whether RheA could act as a repressor in \textit{vivo}, studies were performed in \textit{E. coli}. There is no gene homologous to \textit{rheA} in \textit{E. coli}, and the genes of the small HSP family, \textit{ibpA} and \textit{ibpB}, which are only distantly related to \textit{hsp18}, are under the control of the specific sigma factor \(\sigma^E\). \textit{E. coli} is thus a good host organism in which to study RheA-dependent regulation. The \textit{hsp18} promoter region (−228 to +142) was inserted upstream from the promoterless \textit{bgaB} gene encoding the thermostable \(\beta\)-galactosidase from \textit{Bac. stearothermophilus} in a pUC19 derivative (pPM7002). The \textit{rheA} coding region was cloned behind the \textit{plac} promoter in pSU2719 (pPM7004), a plasmid compatible with ColE1-derived vectors such as pET11a. The strain containing pPM7003, where \textit{rheA} is in the opposite orientation to \textit{plac}, was used as a negative control. Assays of \(\beta\)-galactosidase activity indicated that the presence of RheA decreased expression of \(\beta\)-galactosidase from the \textit{hsp18} promoter by a factor of 24 ([175 versus 4200 Miller units (mg protein)$^{-1}$]), demonstrating that RheA is functional in \textit{E. coli} and specifically regulates \textit{hsp18}.

**Overproduction of RheA in \textit{E. coli}, and DNA-binding studies**

Mobility shift assays were performed to ascertain whether the product of \textit{rheA} interacts directly with the \textit{hsp18} promoter. RheA was overproduced in \textit{E. coli} and crude extracts were prepared from both the control strain (\textit{E. coli} BL21(DE3) carrying pET11a) and the RheA production strain (\textit{E. coli} BL21(DE3) carrying pPS310). Expression of \textit{rheA} in this system was controlled by the IPTG-inducible T7 promoter. A protein of the expected size for RheA (23 kDa) was readily detected by SDS-PAGE of crude extracts in induced cells containing \textit{rheA} (Fig. 1, lane 2). In DNA-binding experiments, extracts of cells of the control strain, without RheA, did not affect the mobility of a 300 bp fragment containing the \textit{hsp18} promoter (Fig. 2, lane 2). In the presence of 0.4 \(\mu\)g of a crude cell extract containing RheA, the labelled fragment was totally retarded (Fig. 2, lane 3), demonstrating the high affinity of RheA for the \textit{hsp18} promoter fragment. In competition experiments to assess the specificity of the binding, the presence of excess unlabelled probe abolished the mobility shift of the labelled \textit{hsp18} promoter fragment (Fig. 2, lane 5), whereas the control with excess \textit{S. albus} \textit{groES} DNA fragment had no effect (Fig. 2, lane 6). These results confirm that RheA binds specifically to the \textit{hsp18} promoter region. Heating (37 \(°C\)) the \textit{E. coli} extracts containing RheA before the gel retardation experiments did not affect the retardation pattern (data not shown).

**Transcription of \textit{rheA} and evidence for autoregulation**

The transcription initiation site of \textit{rheA} was determined by primer extension analysis using RNA isolated from the wild-type strain grown at 30 \(°C\) and subjected to
heat shock for 20 min at 41 °C (Fig. 3). No signal was detected at 30 °C, suggesting that the basal level of rheA transcription is very low. Transcription begins at a cytosine, and the sequence TAACAT at positions −12 to −7 matches the consensus sequence TagPuPuT defined by Strohl (1992) for vegetative promoters in Streptomyces. The ATGCCG at positions −30 to −35 is different from the −35 consensus sequence (TTGACPu). The −10 sequences of the rheA and hsp18 genes partially overlap (Fig. 4), suggesting auto-regulation of RheA.

To test for autoregulation, we analysed the transcription of rheA by Northern blotting. A weak 0.9 kb rheA transcript, detected after temperature upshift in the wild-type strain, could not be detected at 30 °C (Fig. 5). This experiment was repeated with a rheA::tsr mutant (Servant & Mazodier, 1996), in which the rheA gene was disrupted and partially deleted (loss of 148 bp) by the thiostrepton-resistance gene inserted into the region encoding the C-terminal part of the protein. This disruption of rheA results in overproduction of hsp18 mRNA at 30 °C, indicating that the deleted RheA is not functional (Servant & Mazodier, 1996). Analysis of rheA transcription in the rheA::tsr mutant detected a 0.7 kb rheA mRNA in similar amounts at 30 and 41 °C. The 0.7 kb transcript corresponds to the truncated rheA mRNA expected, taking account of the tsr marker in rheA. These results suggest that RheA acts as a repressor not only of the expression of hsp18 but also of its own transcription.

**DISCUSSION**

Although heat-shock induction has been reported for small HSPs in various bacteria, little is known about their regulation. In *Brad. japonicum*, control of the expression of the small hsp multigene family depends on the ROSE sequence, but the mechanism is not yet understood (Münchbach et al., 1999; Naberhaus et al., 1998). Sequences similar or identical to the operator recognized by the CtsR repressor are present upstream from hsp18 of *Clostridium acetobutylicum*, hsp16 of *Streptococcus thermophilus* and hsp18 of *Leuconostoc oenos*; a gene very similar to ctsR is present in *C. acetobutylicum* (Derré et al., 1999b). However, no small HSP regulator has been reported in these bacteria.

We have previously shown that disruption of rheA did not affect expression of genes encoding the main *S. albus* chaperones GroEL, DnaK and ClpB (Servant &
Mazodier, 1996). Here we show that RheA is able to repress expression of the hsp18 gene in E. coli and to bind to the promoter region in vitro, indicating that RheA is the repressor of hsp18. To our knowledge, this is the first report of a repressor that specifically decreases expression of a small HSP in bacteria. In the databases there are currently no genes homologous to rheA, indicating that regulation by RheA is not a widespread regulatory mechanism. For example, rheA is absent from the Mycobacterium tuberculosis genome, although Streptomyces and mycobacteria are taxonomically related and both belong to the order of the Actinomycetales (Cole et al., 1998). The rheA gene might be limited to Streptomyces or a sub-group of Streptomyces. Analysis of a panel of Streptomyces genomic DNA by Southern blotting with a rheA probe and subsequent cloning of the rheA homologues would resolve this issue and help identify a consensus target sequence for RheA.

Fig. 4. Sequence of the rheA and hsp18 promoter regions. The regions encoding hsp18 and rheA are indicated by italic and boxed characters. The transcription start sites are in bold letters, and the −10 and −35 sequences are boxed and shadowed for hsp18 sequences. The ribosome-binding sites (SD) are underlined. Dotted arrows indicate an inverted repeat that may be the target of RheA.

Fig. 5. Northern blot analysis of the S. albus rheA transcripts in the wild-type (a) and mutant (b) rheA strains. Total RNA was extracted from cultures grown for 24 h at 30 °C (lanes 1) and then shifted to 41 °C for 20 min (lanes 2). The probe used was an internal fragment of rheA, amplified by PCR using oligonucleotides XM73 and XM87 (see Methods). The film was exposed by autoradiography for 5 d with an amplifying screen at −80 °C.
repressor might be degraded or might lose its binding capacity at high temperature. The reported experiments do not sustain the second hypothesis but clearly studies of this phenomenon will require a purified functional RheA protein.

In conclusion, in *Streptomyces* regulation of the heat-shock response involves a combination of negative regulators. HspR co-regulates expression of the dnaK operon and the clpB gene. HrcA represses expression of the groEL genes and RheA is the repressor of hsp18. However, other mechanisms will probably be discovered, as at least five groups of HSPs can be defined by the patterns of their induction kinetics after a temperature upshift in *S. coelicolor* (Puglia et al., 1995).

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