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Heat Induction of *hsp18* Gene Expression in *Streptomyces albus* G: Transcriptional and Posttranscriptional Regulation

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In *Streptomyces albus* G, HSP18, a protein belonging to the small heat shock protein family, could be detected only at high temperature. The nucleotide sequence of the DNA region upstream from *hsp18* contains an open reading frame (*orfY*) which is in the opposite orientation and 150 bp upstream. This open reading frame encodes a basic protein of 225 amino acids showing no significant similarity to any proteins found in data banks. Disruption of this gene in the *S. albus* chromosome generated mutants that synthesized *hsp18* RNA at 30°C, suggesting that *orfY* plays either a direct or indirect role in the transcriptional regulation of the *hsp18* gene. In addition, thermally induced expression of the *hsp18* gene is subject to posttranscriptional regulation. In the *orfY* mutant, although *hsp18* RNA was synthesized at a high level at 30°C, the HSP18 protein could not be detected except after heat shock. Synthesis of the HSP18 protein in the *orfY* mutant was also heat inducible when transcription was inhibited by rifampin. Furthermore, when wild-type cultures of *S. albus* were shifted from high temperature to 30°C, synthesis of the gene product could no longer be detected, even though large amounts of *hsp18* RNA were present.

All organisms respond to a sudden increase in temperature by synthesizing members of a small set of proteins called the heat shock proteins (HSPs). HSPs have been highly conserved during evolution, reflecting their important role in cellular metabolism. HSP70 (DnaK), HSP60 (GroEL), and small heat shock protein (smHSP) families are molecular chaperones (12, 16, 21, 25). They are essential for the synthesis, transport, and folding of proteins. Of the major HSPs families, the smHSPs are the least conserved and their function is the most elusive. They form a diverse family of proteins of 15 to 30 kDa which have the tendency to aggregate (1, 28). The common characteristic of smHSPs is the presence of a conserved domain, often referred to as the α crystallin domain, which constitutes the C-terminal halves of the proteins (8). The family includes the α crystallin protein of the vertebrate eye and is found in several prokaryotes, including *Clostridium acetobutylicum*, *Mycobacterium tuberculosis*, and *Stigmatella aurantiaca* (18, 31, 39). In eukaryotes, smHSPs are among the strongly induced HSPs, and in addition, the smHSP genes can also be induced (in the absence of stress) during particular stages of development (1). In *Stigmatella aurantiaca*, smHSP SP21 is not present in vegetative cells but is in spores (18). In mycobacteria, a stress protein of 18 kDa is a major immunogen of the immune response (2). The smHSPs display chaperone functions in vitro (6, 20, 21) and have a role in thermotolerance in mammalian cells (23) but not in yeast cells (29).

Recently, a smHSP, HSP18, has been identified in *Streptomyces albus* G (33). *Streptomyces* spp. are gram-positive mycelial soil bacteria that undergo a complex cycle of morphological differentiation leading to sporulation. Disruption mutagenesis of *hsp18* showed that HSP18 is not essential for growth between 30 and 42°C but is involved in thermotolerance. A homologous *hsp18* gene has been detected by Southern blotting in different *Streptomyces* spp. like *S. parvulus* and *S. pristinaespiralis* but not in *S. lividans* or *S. coelicolor* (unpublished results). Transcription of *hsp18* is induced by heat shock, and no *hsp18* mRNA could be detected at 30°C.

Regulation of heat shock genes in *Escherichia coli* is well characterized; in this bacterium, specific sigma factors, σ^{32} and σ^{24}, control the expression of the heat shock regulon (reviewed in references 5 and 44). These sigma factors are required for recognition of specific heat shock promoters by the RNA polymerase, and transcription of heat shock genes is controlled by the level and activity of σ^{32}. In *Bacillus subtilis*, there are at least three classes of heat shock genes that are regulated by different mechanisms. The regulation of class I heat shock genes involved an inverted repeat structure, called CIRCE (controlling inverted repeat of chaperone expression). CIRCE has been identified upstream of the *groE* and *dnaK* genes of many bacteria (see Table 1 in the review by Hecker et al. [17]). In *B. subtilis*, CIRCE serves as an operator for a repressor encoded by *hrcA*, the first gene of the *dnaK* operon (32, 43). Class II genes, of which there are more than 40, are regulated by the alternate sigma factor, σ^B. Class III heat shock genes can respond independently of σ^B or CIRCE.

The heat shock response has been also studied in two *Streptomyces* species, *S. coelicolor* and *S. albus. Streptomyces* spp. contain two *groEL* genes which are transcribed from vegetative σ^{70}-like promoters (9, 15). Synthesis of *groEL* mRNA is induced by heat shock. A CIRCE motif present in the promoter region could be the binding site for a regulatory protein. In *S. albus*, the *groEL1* gene is also subject to posttranscriptional regulation (35). Transcription of the *dnaK* operon of *S. coelicolor* is heat inducible. The *dnaK* operon contains four genes, 5′ *dnaK-grpE-dnaJ-hspR*. The *S. coelicolor* HspR protein is able to interact specifically with the *dnaK* operon promoter, suggesting that it may be a regulator (4). Patterns of gene expression in *S. coelicolor* following heat shock during growth have been analyzed by two-dimensional protein electrophoresis (30). Five groups of HSPs were defined by their kinetics of induction after the temperature shift and changes of expression during growth phase. No smHSPs were detected in *S. coelicolor*. These results suggest a combination of regulatory factors which allow differential expression according to the growth phase and the duration of the heat shock.

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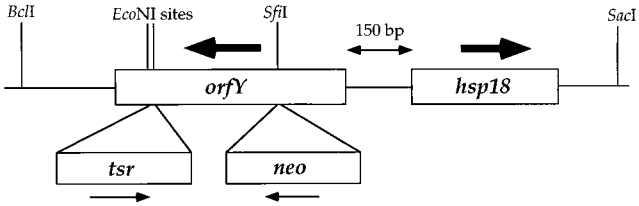

FIG. 1. Restriction map and genetic organization of the *hsp18* and *orfY* genes of *S. albus.*

We investigated the regulation of the *hsp18* gene in *S. albus*. Under the conditions employed, the HSP18 protein, like GroEL, is constitutively synthesized at high temperature in contrast to DnaK, whose induction is transient. Furthermore, *hsp18* transcription is induced by heat shock, but there are no CIRCE sequences upstream. These data suggested that the regulation of *hsp18* is different from that of *dnaK* and *groEL* genes. In this work, we show that *orfY*, located upstream from *hsp18*, is involved in its transcriptional regulation. In addition, *S. albus hsp18* gene expression is also subject to posttranscriptional regulation.

MATERIALS AND METHODS

Bacterial strains, media, plasmids, and bacteriophages. *S. albus* G strain J1074, which is defective for both *Sal*I restriction and modification systems (7), was obtained from the John Innes Culture Collection and grown in yeast extract-malt extract rich liquid medium (19). NE was used for growth of *Streptomyces* spp. on plates (19). Antibiotics were added to a final concentration of 25 μg/ml for thiostrepton, 30 μg/ml for viomycin, and 250 μg/ml for hygromycin. *E. coli* TG1 (13) was used as the general cloning host, and *E. coli* S17-1 (36) carrying an integrated RP4 derivative was used for intergeneric conjugation from *E. coli* to *S. albus. E. coli* strains were grown in Luria-Bertani broth supplemented with ampicillin (100 μg/ml) or hygromycin (200 μg/ml) when appropriate. pUC18, pUC19, and pHM11a have been described previously (26, 41). M13mp18 and M13mp19 derivatives generated for sequencing were propagated in *E. coli* TG1 (41).

DNA manipulation and sequencing. Standard methods were used for plasmid construction and transformation of *E. coli* for cloning (24). Both strands were sequenced by the chain termination method of Sanger et al. with the M13 universal primer using T7 polymerase (T7 sequencing kit; Pharmacia). 7-deaza-dATP was used to minimize band compression. Part of the sequence was also determined with synthetic oligonucleotide primers designed on the basis of known sequences.

Isolation of RNA. Total RNA from *S. albus* was prepared as described previously (35). RNA was stored in H_2O plus 0.1% diethyl pyrocarbonate at −20°C and quantified by measuring the optical density at 260 nm. The quality of the RNA was checked for each preparation by electrophoresis on 1.4% agarose gels.

Northern (RNA) blot analysis. Equal amounts of RNA (20 μg) were separated by electrophoresis (1.4% agarose–6.8% formaldehyde–1× MOPS [morpholinepropanesulfonic acid] buffer) and transferred to a nitrocellulose filter (Hybond N membrane; Amersham) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight. Nucleic acids were fixed by UV irradiation. Hybridization was performed at 65°C in 5× SSC–5× Denhardt's solution–0.5% sodium dodecyl sulfate (SDS)–4 mM EDTA. Blots were washed with 0.5× SSC–0.1% SDS at 65°C. The probe used corresponds to the *hsp18* gene coding region (437-bp *Kpn*I-*Pst*I fragment of pPM1771 [33]).

Plasmid construction. pPM1787, used to disrupt the *orfY* gene in the region encoding the C terminus, was constructed as follows. A *Hin*dIII-*Bam*HI fragment of pPM1745 (33) was inserted between the *Hin*dIII and *Bam*HI sites of pUC18 to yield pPM1781. The insert contains *orfY* plus 700 bp downstream and 245 bp upstream. The *tsr* gene (37) was isolated on a 1.8-kb *Bam*HI fragment from pIJ39, treated with T4 DNA polymerase to produce blunt ends, and inserted between the *Eco*NI blunted sites of pPM1781 to obtain pPM1785. pPM1787 was constructed by ligation of *Hin*dIII-digested DNA of plasmid pPM1781 with the *Hin*dIII fragment containing the viomycin *oriT* cassette (34). This cassette allowed both transfer of the plasmid from *E. coli* to *S. albus* and screening for double-crossover events.

pPS217, used to disrupt most of the *orfY* gene, was constructed as follows. A *Xba*I-*Bam*HI fragment of pPM1745 (33) was cloned between the *Xba*I and *Bam*HI sites of pUC19 to obtain pPS214 containing the complete *orfY* gene and a part of *hsp18*. pPS215 was constructed by subcloning the *vio oriT* cassette into the unique *Hin*dIII site of pPS214. To disrupt the *orfY* gene, pPS215 was treated with *Sfi*I and then blunted. This linearized plasmid was then ligated with the 2.3-kb *neo* cassette from pHP45Ω-Km (10) digested by *Bam*HI and blunted to generate pPS216. pPS217 was obtained by subcloning the *Mlu*I-*Eco*RI fragment of pPM1701 (33), which contains the 3′ part of *hsp18* and downstream region, into the pPS216 digested by *Mlu*I-*Eco*RI.

To complement the *orfY* mutant, *orfY* was cloned in an expression vector, pHM11a (26). To obtain a DNA fragment corresponding only to the *orfY* coding region, a PCR was carried out with a pair of oligonucleotides (PS90, GTGGA TCCTCAGGACCGCCCGGAC; PS92, TGTGATCATATGACCACCGCCGA CCGCCC). The *Nde*I-*Bam*HI-digested PCR fragment was cloned into *Nde*I-*Bam*HI-treated pUC19, leading to pPS310. The *Nde*I-*Bam*HI fragment of pPS310 was subcloned into the *Nde*I-*Bam*HI-digested pHM11a to yield pPS250.

Heat shock response in *Streptomyces* spp. and gel electrophoresis. Approximately 10^5 spores were used to inoculate yeast extract-malt extract liquid medium, and cultures were grown for 24 h at 30°C. One-milliliter aliquots of culture were either labeled immediately for 10 min with 80 μCi of [^{35}S]methionine-cysteine (ICN) at 30°C or shifted to 41°C for various times and then labeled for 10 min. Three milliliters of ice-cold buffer A (20 mM Tris HCl [pH 7.5], 2 mM EDTA, 1 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg of benzamidine per ml, 5 μg of leupeptin per ml) was added. The cells were collected by centrifugation, resuspended in 200 μl of buffer A, and stored at −70°C. Thawed cell suspensions were disrupted by sonication, and SDS was added to a final concentration of 0.3%. Samples were then heated to 85°C for 5 min and centrifuged at 12,500 × *g* for 15 min. The supernatants were stored at −20°C.

Equal amounts of total protein extracts (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) as described by Laemmli (22).

Nucleotide sequence accession number. The DNA sequence of *orfY* has been deposited in GenBank under accession number U59153.

RESULTS

Nucleotide sequence of the region located upstream of *hsp18*. pPM1701 is pUC18 carrying a 2.9-kb *Bcl*I-*Sac*I *S. albus* DNA fragment which contains the *hsp18* gene encoding a smHSP (33). A 1,000-bp DNA fragment upstream from the *hsp18* gene was sequenced. There was an open reading frame (*orfY*) in the opposite orientation to the *hsp18* gene (Fig. 1). *orfY* encodes a 225-amino-acid protein with a size of 23,131 Da (with a pI of 9.63 predicted by the Genetics Computer Group software program). *orfY* (Fig. 2) exhibits the biased codon usage characteristic of the G+C-rich coding DNA in *Streptomyces* genomes (40). The product of *orfY* has no significant similarity to any proteins present in databases. The most likely

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                                                    hsp18
                                                     <<<
ACTCGCGGAAGGGGTCAGTGCGCATCAGCATGAGTGAACACCTCCAGAAGTCCAGGCAGT  60
TGATGCCATGCGCTTCGCTTGGCACCGTTGTAACATGTCATCCGAACGATGACAAGCGAG 120
TCGTCGTCCATCGGATGACGACCCGGAGTGCGCGACACGGCCGCGCGGCACAGGAGGCCC 180
 >>>
CATGACCACCGCCGACCGCCCCACCCCCGGCCCCACCGGACGGGTCGGCCCGCCCTTCAC 240
  M  T  T  A  D  R  P  T  P  G  P  T  G  R  V  G  P  P  F  T
CCCCGACCCGGCCTCCTTCCTGGCCGTCGCGGGCGCCCTGGAGGCCATCCGGGACGCCGT 300
  P  D  P  A  S  F  L  A  V  A  G  A  L  E  A  I  R  D  A  V
CGACGCCGCCGAGTCCGGCACAGCCGGGAGCGGCGGCGGGGGAGCGGGGGCCGAGGACGT 360
  D  A  A  E  S  G  T  A  G  S  G  G  G  G  A  G  A  E  D  V
CCTCGCCAGCCTGCTCCTGCTGCGCCAACTGCGGGAGCAGCTCGCCGTCTGGGAACCCGG 420
  L  A  S  L  L  L  L  R  Q  L  R  E  Q  L  A  V  W  E  P  G
CCTCGTCGAGACGGCCCGCGAGGCGGGCGCCAGCTGGGCCGAGCTGGCCCACCCCCTGGG 480
  L  V  E  T  A  R  E  A  G  A  S  W  A  E  L  A  H  P  L  G
CGTCGCCAGCCGCCAGGCCGCCGAACGCCGCTACCTGCGCAACCGCCCCGGCGCCTCGGG 540
  V  A  S  R  Q  A  A  E  R  R  Y  L  R  N  R  P  G  A  S  G
CACCACCGGCGAACAGCGCGTCCAGGCCACCCGCGAACGCCGCGCCGCCGACCGCGACCG 600
  T  T  G  E  Q  R  V  Q  A  T  R  E  R  R  A  A  D  R  D  R
CACCGCCTGGGCCCGGCGCAACGCCGCCGACCTGCGCCGCCTGGCCGCCCGCCAGATCACCAG 660
  T  A  W  A  R  R  N  A  A  D  L  R  R  L  A  A  Q  I  T  S
CCTCACCGGCCTGCCCGCCGCCGCCGCCCGCCTCCCCGTCGCCCGCCTCGACCAGGCCCTCGC 720
  L  T  G  L  P  A  A  A  R  L  P  V  A  R  L  D  Q  A  L  A
CCACGACGACCCGGCCGCCCTCCTCGGCCCCCTCCGCGAGGCCGCGCCCCCGCTCCGCTC 780
  H  D  D  P  A  A  L  L  G  P  L  R  E  A  A  P  P  L  R  S
CGCCCACCCCGAACTGGCCGGCCGGATCGACGCCCTGTCCGGCCCCGACGGCACGGCCTC 840
  A  H  P  E  L  A  G  R  I  D  A  L  S  G  P  D  G  T  A  S
GTCCGGGCGGTCCTGACCCGCGCCCGGCGCTCTCGCGTGCGCGGCGGACCCCCGTACCGC 900
  S  G  R  S  *                                    ──────────
AGACTGCGGCTGTGACAGAGCGGCGGCAGCGGTTCGAGGGGTGGATCGCCGGGGTCGGGA 960
──────►                ◄──────────
CCGGCTCCGGTACGCGACTGGTGCTCGGGCACTGGGAGCGGTCGCCGTTCGGCGCGTTCA 1020
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FIG. 2. Nucleotide and deduced amino acid sequences of the 1,020-bp segment of DNA containing *orfY*. The putative start codon (underlined) is shown. A plausible ribosome binding site for *orfY* is indicated in bold characters. The inverted arrows at the end of the sequence indicate a potential terminator site for the RNA polymerase 32 bp downstream from the TGA (*) termination codon. The region encoding HSP18 is indicated by italic bold characters.

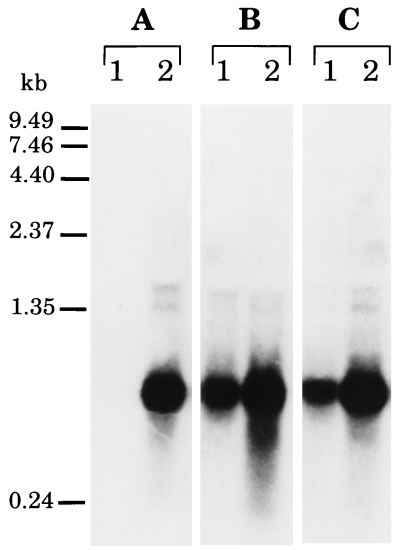

FIG. 3. Northern blot analysis of the *S. albus hsp18* transcripts in the wild-type strain (A), the *orfY*::*tsr* mutant (B), and the *orfY*::*neo* mutant (C). Total RNA was extracted from cultures grown for 24 h at 30°C (lanes 1) and then shifted to 41°C for 20 min (lanes 2). The probe used corresponds to the *hsp18* structural gene (fragment *Kpn*I-*Pst*I of pPM1771).

start codon is the ATG at nucleotide 182, which is preceded, 4 nucleotides upstream, by a potential ribosome binding site (AGGAGG). The start codons of the *hsp18* and *orfY* genes are therefore separated by only 150 bp. An imperfect inverted repeat, 32 nucleotides downstream of the *orfY* stop codon, is capable of forming a secondary structure with a ΔG of -23.6 kcal/mol (ca. -98.7 kJ/mol) and which may be a transcription terminator.

Analysis of *hsp18* gene expression in two different *orfY* mutants. To investigate the role if any of *orfY* in the expression of the *hsp18* gene (or other HSPs), the gene was disrupted and partially deleted (148 bp) by introducing the thiostrepton resistance gene at the region encoding the C terminus. An *E. coli-Streptomyces* sp. conjugative vector (34) was used to disrupt *orfY*. The *orfY* gene in vitro was disrupted by insertion of a *tsr* cassette between the *Eco*NI sites 100 and 148 bp upstream from the stop codon into the pPM1787 plasmid (Fig. 1). After conjugation of *E. coli*(pPM1787) and *S. albus*, thiostrepton-resistant conjugants were obtained and screened for viomycin sensitivity. Tsr^r Vio^s colonies were assumed to have resulted from gene replacement events (double crossover) and to carry the *orfY* mutation (6% of double recombinants were obtained). Chromosomal DNA was isolated from several independent Tsr^r Vio^s transconjugants and analyzed by Southern hybridization to confirm *tsr* integration by double crossover in the *orfY* region (data not shown).

The resulting *orfY*::*tsr* mutant was tested by Northern (RNA) blotting for expression of the *hsp18* and *groEL2* genes and the operons *groES-groEL1* and *dnaK* (data shown only for *hsp18*). Disruption of *orfY* resulted in expression of *hsp18* mRNA (500 b) at a high level at 30°C (Fig. 3, lane B1). In the wild-type strain, no *hsp18* mRNA was detected at 30°C (lane A1). In the *orfY*::*tsr* mutant, the amount of *hsp18* mRNA was also increased after heat shock (two- to threefold). The mutation had no effect before or after heat shock on the levels of *groES-groEL1*, *groEL2*, or the *dnaK* operon mRNA (data not shown).

Although the C-terminal part of OrfY was disrupted, a truncated OrfY could keep a weak activity. This partial activity could explain the small increase of *hsp18* mRNA at high temperature. To confirm or rule out this possibility, another mutant, *orfY*::*neo*, was constructed. The *orfY* gene was disrupted by insertion of a neomycin resistance cassette into the unique *Sfi*I site located at the 5′ end of *orfY* (280 bp downstream of an ATG codon, into the pPS217 plasmid) (Fig. 1). After conjugation of *E. coli*(pPS217) and *S. albus*, neomycin-resistant conjugants were obtained and screened for viomycin sensitivity. Six percent of double recombinants were obtained, and integration of *neo* into the genome was confirmed by Southern hybridization (data not shown). The *orfY*::*neo* mutant was analyzed by Northern blotting for expression of the *hsp18* gene. *hsp18* mRNA was expressed at a high level at 30°C (Fig. 3, lane C1), and the amount of *hsp18* mRNA was also slightly increased after heat shock in the same way as it was in the *orfY*::*tsr* mutant.

These results suggest that disruption of *orfY* eliminates a factor responsible, either directly or indirectly, for the negative transcriptional regulation of *hsp18*. The weak increase in the amount of *hsp18* mRNA after heat shock in the *orfY*::*tsr* mutant was not due to a partial activity of OrfY.

Complementation of the *orfY*::*tsr* mutant. Disruption of *orfY* on the chromosome of *S. albus* causes transcriptional activation of *hsp18* at 30°C. To rule out that the observed characteristics of the *orfY* mutant were due to a polar effect of the insertion of *tsr* on genes downstream of *orfY*, we complemented the mutant by adding in *trans* a functional copy of *orfY*. In integrative plasmid pPS250, the expression of the *orfY* gene is under the control of a strong constitutive promoter, P_E (promoter up-mutant derived from the wild-type promoter of the erythromycin resistance gene of *Saccharopolyspora erythrea*). The *orfY*::*tsr* mutant was transformed with pPS250 or, as a control, with pHM11a. The resulting strains were grown at 30°C or submitted to a heat shock, and the level of mRNA *hsp18* was investigated. Figure 4 shows that the level of *hsp18*

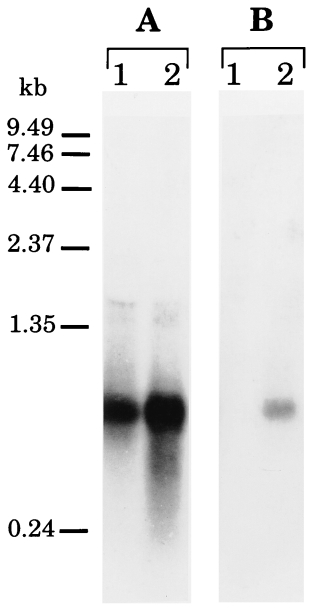

FIG. 4. Complementation of the *orfY*::*tsr* mutant by the OrfY-overproducing plasmid pPS250. Total RNA was extracted from a culture of the *orfY*::*tsr* mutant with control plasmid pHM11a (A) or with plasmid pPS250 (B) grown at 30°C (lanes 1) or submitted to heat shock for 20 min at 41°C (lanes 2). For the Northern blot, RNAs were hybridized with an *hsp18*-specific probe.

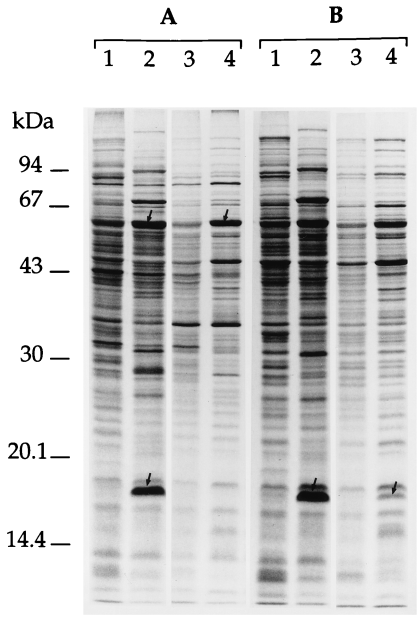

FIG. 5. Expression of HSP18 in the *orfY S. albus* mutant and effect of rifampin on the production of heat shock proteins. Aliquots (1 ml) of a culture grown at 30°C were either labeled immediately for 10 min (lanes 1) or shifted to 41°C for 10 min and then labeled for 10 min (lanes 2). Rifampin (300 μg/ml) was added, and the samples were incubated at 30°C for 3 min and either labeled immediately for 10 min (lanes 3) at 30°C or shifted to 41°C for 3 min and labeled for 10 min (lanes 4). The amount of dimethyl sulfoxide used to solubilize rifampin did not induce expression of HSPs. Total proteins were extracted from *S. albus* wild-type (A) and *orfY*::*tsr* mutant (B) strains and separated by SDS-PAGE (12.5% polyacrylamide). HSP18 and GroEL are indicated by arrows. Gels were exposed for 3 days to X-ray film for rifampin experiments.

mRNA is at the wild-type level at 30°C in *orfY*::*tsr* transformed with pPS250. The amount of *hsp18* mRNA was increased after heat shock but less than that in the wild type. The overexpression of *orfY* under the control of P_E can explain this phenomenon.

Production of HSP18 protein in the wild-type and *orfY*::*tsr* mutant strains: evidence for a posttranscriptional regulation. To verify whether the overproduction of mRNA was accompanied by an increased HSP18 level, the synthesis of the HSP18 protein was visualized by labeling the proteins at 30°C or after a temperature upshift (Fig. 5). Surprisingly, no HSP18 was detected in the *orfY*::*tsr* mutant at 30°C (Fig. 5, lane B1), although the *hsp18* mRNA was present at this temperature (Fig. 3, lane B1). HSP18 synthesis in the mutant was indistinguishable from that of the wild type both before and after heat shock. This result suggests that thermoregulation of *hsp18* depends not only on transcriptional control but also on posttranscriptional control. We tested the effect of rifampin, a specific inhibitor of transcription, on the induction of HSP18 production. Northern blot analysis showed that *hsp18* RNA synthesis was blocked 2 min after the addition of rifampin, and no induction of mRNA synthesis was observed after temperature upshift (data not shown). [^{35}S]methionine-cysteine labeling experiments with wild-type *S. albus* showed that the HSP18 protein was not produced after temperature upshift in the presence of rifampin (Fig. 5, lane A4), in accordance with the fact that no *hsp18* mRNA was produced in these conditions. In contrast, in the *orfY*::*tsr* mutant, labeling experiments in the presence of rifampin showed that the HSP18 protein was produced only at 41°C (Fig. 5, lane B4). Thus, the *hsp18* mRNA present at 30°C is translated only when the temperature increases, evidence for a posttranscriptional thermoregulation. In this experiment, we also observed thermally induced synthesis of GroEL (56- to 58-kDa) proteins in the presence of rifampin, as described previously (35).

Effect of a shift back to 30°C on HSP18 synthesis. To confirm posttranscriptional regulation of *hsp18* expression, a [^{35}S] methionine-cysteine labeling experiment was repeated but after a shift back to 30°C (Fig. 6). The kinetics of HSP synthesis were then analyzed. Several profiles of induction were observed: a transient induction of HSP90 and HSP70 (DnaK) and a constitutive high expression for GroEL (56-58 kDa) and HSP18 proteins at high temperature. When the cultures were shifted back to 30°C, although *hsp18* mRNA was present, synthesis of HSP18 fell strongly after 3 min and was undetectable 5 min after the downshift. *hsp18* mRNA was still present 10 min after the downshift (data not shown). These results confirm that *hsp18* is subject to posttranscriptional regulation and that high temperature is required for its expression.

Stability of *hsp18* mRNA. To determine whether the decrease of HSP18 synthesis observed after a shift back to 30°C is due to nontranslation of the mRNA or to rapid degradation of the mRNA, the decay of *hsp18* mRNA was monitored at 30 and 41°C. The decay of *hsp18* mRNA (500 b), at 30 and 41°C, was monitored after adding rifampin after 30 min of heat shock (Fig. 7). Twenty minutes after the shift back to 30°C, *hsp18* mRNA was still present. This indicates that the decrease in the rate of HSP18 synthesis after a shift back to 30°C is a translational effect and not due to rapid degradation of *hsp18* mRNA. The intensities of the radioactive signals corresponding to the *hsp18* transcripts were quantified with a PhosphoImager. The half-life of *hsp18* mRNA at 30°C (i.e., 10 min) was shorter than that at 41°C (i.e., 15 min). The stability of the HSP18 protein was also monitored at 30 and 41°C by pulse-chase experiments (data not shown). The HSP18 protein was equally stable at 30 and 41°C, ruling out the possibility that the observed decrease of HSP18 synthesis after a shift back to 30°C was due to rapid specific degradation of HSP18.

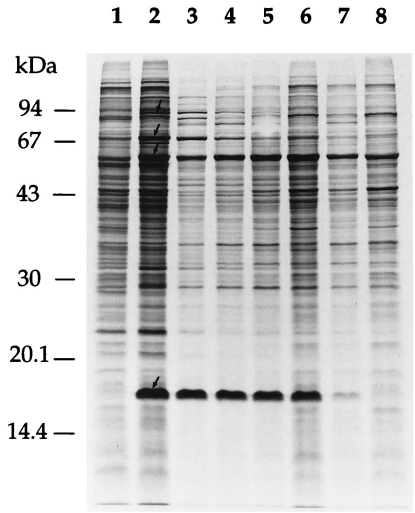

FIG. 6. Effect of a shift back to 30°C on HSP synthesis in *S. albus* wild type. Cultures (20 h after inoculation) growing at 30°C were either labeled immediately for 15 min at 30°C (lane 1) or shifted to 41°C for 5, 20, 40, 60, or 120 min (lanes 2 to 6, respectively) and then labeled for 15 min at 41°C. After induction of 120 min at 41°C, a culture was shifted back to 30°C and similarly labeled 3 min (lane 7) and 5 min (lane 8) later. HSP90, HSP70 (DnaK), HSP56-58 (GroEL), and HSP18 are indicated by arrows.

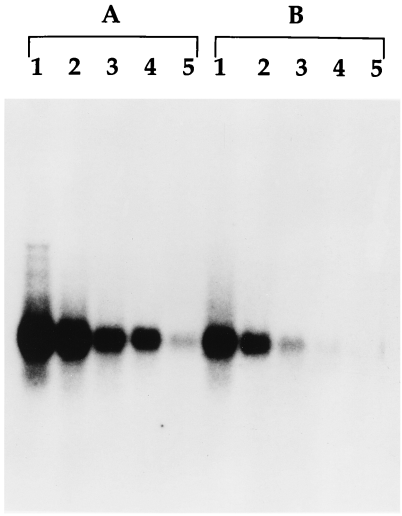

FIG. 7. Stability of *hsp18* mRNA in *S. albus* wild type. In parallel, two cultures (20 h after inoculation) growing at 30°C were shifted to 41°C for 30 min to induce heat shock genes. After 30 min, rifampin (300 μg/ml) was added to block transcription. One culture was left at 41°C (A), and the other was shifted back to 30°C (B). RNA was extracted at 0, 10, 20, 30, and 40 min after rifampin addition (lanes 1 to 5, respectively). The probe used corresponds to the *hsp18* structural gene (fragment *Kpn*I-*Pst*I of pPM1771).

DISCUSSION

Expression of *hsp18* is induced by heat shock at the mRNA level. The transcription initiation site of *hsp18* is preceded at positions −10 and −35 by sequences similar to hexamers of the streptomycete vegetative promoter (33). We demonstrate that the *orfY* gene upstream from and in the opposite orientation to the *hsp18* gene plays a direct or indirect role in the transcriptional regulation of the *hsp18* gene. The *orfY* mutant produced a high level of *hsp18* mRNA at 30°C with a moderate increase when grown at high temperature. This increase could be due to two phenomena: (i) differential stability of mRNA at 30 and 41°C and (ii) a second induction mechanism. The transcription of the *hsp18* gene could be controlled by two different mechanisms: negative regulation involving the *orfY* effector and a second induction mechanism independent of *orfY*. The heat shock regulation of smHSPs in bacteria remains poorly characterized. In *C. acetobutylicum*, the gene located upstream from the smHSP (*hsp18*) encodes a $tRNA^{Thr}$ (31). The promoter is identical to the consensus promoter of gram-positive bacteria, and no CIRCE structure was detected upstream.

A model in which transcription of *hsp18* is repressed at 30°C by association of the *orfY* product with a DNA motif to block transcription can be envisaged. In the 150-bp DNA region which separates the *hsp18* and *orfY* genes, two inverted repeats are present: one is in the promoter region of *hsp18*, and the other is 60 bp from the *orfY* initiation codon and could be the target of a repressor. In the promoter region of *hsp18*, the sequence GTCATC-5N-GATGAC corresponds to a perfect inverted repeat; 60 bp from the *orfY* initiation codon, a similar but imperfect inverted repeat was found, GTCGTC-5N-GATGAC. Proteins binding in the promoter region, particularly between −10 and −35 hexamers, are known to have repressor activity (14). The binding of a repressor near the −35 region of *hsp18* could interfere with binding of the RNA polymerase and thereby cause the repression. In *S. albus*, the regulation of the heat shock response seems to involve a combination of regulators. *hspR* represses the expression of *dnaK* (13a), and the CIRCE motif upstream from the *groEL* genes seems to be the target of a regulatory protein (9). Each regulator is specific and controls the expression of one or several heat shock genes. In the *orfY* mutant, the heat shock response of DnaK and GroEL is not affected. Therefore, in *S. albus*, there are at least three classes of heat shock genes with respect to the type of regulation.

We will attempt to elucidate the complete regulation pathway for the *hsp18* gene and, in particular, whether the product of *orfY* interacts directly or indirectly with *hsp18*. The overproduction of the product of *orfY* in *E. coli* and DNA binding studies should answer this question and reveal the DNA target sequence. In contrast to most prokaryotes in which HSPs are transiently induced, the HSP18 protein is expressed continuously at high temperature. Possibly, the repressor is unable to function or is unstable at high temperature.

hsp18 expression was also posttranscriptionally regulated. Although *hsp18* mRNA was found at 30°C in the *orfY* mutant, no HSP18 protein was synthesized. In *Streptomyces* spp., the use of a rare tRNA (encoded by the *bldA* gene) needed for the translation of a leucine codon (TTA) controls the expression of various genes containing this codon (11). The TTA, TTT, and CTA codons, which are the rarest codons in *Streptomyces* spp., do not appear in the sequence of the *hsp18* gene. Therefore, the use of a rare tRNA is unlikely to be the mechanism of posttranscriptional regulation of the *hsp18* gene.

Posttranscriptional regulation could occur at three levels: (i) in the translation of mRNA, (ii) in the stability of the transcript, and (iii) in the stability of the product. Our experiments indicate that translational control is the most probable. The stability of the HSP18 protein was not affected by temperature. The stability of mRNA was slightly lower at 30°C than at 41°C but not low enough to explain the absence of production of HSP18 at 30°C by the *orfY* mutant. Translation frequency and mRNA stability are positively correlated because a more-dense packing of ribosomes on mRNA would sterically protect against nucleolytic attack (42). Ribosomes protect mRNAs from nuclease degradation, and this could explain the difference in *hsp18* mRNA stability at 30 and 41°C. Translational regulation may depend on either the translational machinery or the secondary structure of the mRNA. The first hypothesis is in accordance with the theory suggested by VanBogelen and Neidhardt, i.e., that the ribosome is implicated as a direct sensor in the control of the expression of heat and cold shock genes (38). Brandi et al. showed that the ribosome itself might acquire an increased capacity to translate a *cspA* mRNA when induced by cold shock (3). It seems more likely that the translational control of *hsp18* involves mRNA secondary structures that may prevent binding of the ribosome to the translational initiation region of the *hsp18* mRNA at 30°C. Under heat shock conditions, the secondary structure responsible for translational repression may be destabilized, making the translation initiation region available to ribosomes. This mechanism has been described in *E. coli* for the heat induction of σ^{32}, a sigma factor specific for the heat shock promoters (27, 45). In *S. albus*, the *groEL1* gene is subject to posttranscriptional regulation (35). The regulatory signals needed for heat induction were mapped within the *groEL1* structural gene. The regulation of *hsp18* may involve this mechanism. Studies of translational fusions between *hsp18* and a reporter gene should allow the localization of the sequence involved in the posttranscriptional regulation of *hsp18*.

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