

## *hrcA*, Encoding the Repressor of the *groEL* Genes in *Streptomyces albus G*, Is Associated with a Second *dnaJ* Gene

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Received 9 March 1998/Accepted 3 August 1998

**Expression of the principal chaperones of the heat shock stimulon of *Streptomyces albus G* are under the negative control of different repressors. The *dnaK* operon is regulated by *hspR*, the last gene of the operon (*dnaK-grpE-dnaJ-hspR*). *hsp18*, encoding a member of the small heat shock protein family, is regulated by *orfY*, which is in the opposite orientation upstream of *hsp18*. The *groES-groEL1* operon and the *groEL2* gene are regulated differently. They present tandem copies of the CIRCE element found in the 5' region of many heat shock genes and shown to act in *Bacillus subtilis* as an operator for a repressor encoded by *hrcA* (*hrc* stands for heat regulation at CIRCE). We report the identification in *S. albus* of a new heat shock operon containing *hrcA* and *dnaJ* homologs. Disruption of *hrcA* increased the transcription of the *groES-groEL1* operon and of the *groEL2* gene. These features were lost when the mutant was complemented in *trans* by an intact copy of *hrcA*. Despite considerable accumulation of the GroE chaperones in the *hrcA* mutant, there was no effect on formation of the aerial mycelium and sporulation, indicating that neither *hrcA* nor the level of *groE* gene expression is directly involved in the regulation of *Streptomyces* morphological differentiation.**

*Streptomyces* spp. are filamentous soil bacteria belonging to the high-G+C branch of the gram-positive eubacteria. These bacteria have a complex morphological cycle. On solid media, spores germinate and form a basal mycelium. This is followed by aerial mycelium development, and septation of the mycelium leads to spore formation. *Streptomyces* spp. are metabolically active during the stationary phase and produce a very large variety of secondary metabolites. We are interested in studying the heat shock response in *Streptomyces* because heat shock proteins (HSPs) may be involved in physiological and morphological differentiation. Nutritional imbalances activate a developmental program involving the heat shock stress regulon (33). This suggests that the regulatory systems that control HSPs are involved in *Streptomyces* development. We are also interested in identifying the principal HSPs of *Streptomyces*, because they could be used to facilitate protein overproduction and secretion (45). Manipulation of the *hsp* genes may make it possible to develop *Streptomyces* hosts that produce homologous or heterologous proteins more efficiently.

Scores of differentiation mutants have been obtained in *Streptomyces* in recent years. They fall into two main classes: "bald" mutants, unable to produce aerial mycelia, and "white" mutants, unable to sporulate (4). Several mutations have been characterized, but none are related to the *hsp* genes. However, the cascade of events leading to the change from basal mycelium to aerial mycelium formation has not been elucidated.

The heat shock response and its regulation were first studied in detail in *Escherichia coli*. There are two heat shock regulons, positively regulated at the level of transcription by sigma factors. The largest regulon is controlled by  $\sigma_{32}$ , and the smallest regulon is controlled by  $\sigma_{24}$  (for recent reviews see references 3, 11, and 48).

However, recent results obtained with other bacteria show a different situation. In *Bacillus subtilis*, the heat shock response of which has also been studied in detail, three classes of heat

shock genes have been described. The number of classes is likely to increase because class III comprises heat shock genes of unknown regulation. The chaperone-encoding genes *dnaK* and cohort *dnaJ* and *grpE* and the chaperonin-encoding genes *groEL* and *groES* are ubiquitous. These are class I genes in *B. subtilis*, and their thermoregulation depends on a repressor and an operator CIRCE (for controlling inverted repeat of chaperone expression) (35, 49). CIRCE elements have been identified more than 50 times in 28 eubacterial species (17). CIRCE has been reported to be associated with the *dnaK* and/or *groEL* genes or their cohort, *grpE*, *dnaJ*, and *groES*. In *B. subtilis*, the gene of the *dnaK* operon furthest upstream, formerly called *orf39*, has been shown to encode the repressor of the *dnaK* and *groEL* operons. This protein binds to CIRCE-bearing DNA fragments (47), and *orf39* has been renamed *hrcA* (for heat regulation at CIRCE).

It is likely that all microorganisms with CIRCE contain a gene similar to *hrcA*. Genes similar to *hrcA* have been identified by chance by sequencing the *dnaK* operon and during the sequencing of the entire genomes of organisms.

In *Streptomyces* spp., neither *hrcA* nor CIRCE is associated with the *dnaK* operon, the regulation of which depends on another repressor, HspR (2, 13). CIRCE is associated with two *groEL* genes (*groEL1* and *groEL2*) (25). *groEL1* forms an operon with a *groES* gene, whereas *groEL2* maps to another location on the chromosome (7, 34) and is not associated with a *groES* gene. Transcription of these genes is thermoregulated. Transcription of the *groES-groEL1* operon generates two mRNAs: a 500-base *groES* transcript and a 2,200-base polycistronic *groES-groEL1* transcript. The transcript of *groEL2* is 1,900 bases long (15). Two copies of CIRCE are present in tandem upstream from *groESL1* and *groEL2*. One copy is located between the transcriptional and translational start sites, and the other overlaps the -35 hexamer of the promoter (7). The presence of CIRCE elements and the detection of a CIRCE DNA-binding protein in gel retardation assays (8) suggests that an *hrcA*-like gene is present in *Streptomyces*.

We report here the cloning and characterization of the *Streptomyces albus hrcA* gene. The effects of disrupting *hrcA* show that it is the principal regulator of *groEL* expression and

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that neither *hrcA* nor the level of *groEL* is directly involved in control of the *Streptomyces* cell cycle.

## MATERIALS AND METHODS

**Bacterial strains, media, and plasmids.** The *S. albus* G strain (J1074), deficient in both *Sall* restriction and modification systems (5), was obtained from the John Innes Culture Collection (Norwich, England). *S. albus* was routinely cultivated on NE plates (29) or in YEME rich liquid medium (20). When required, thio-strepton and viomycin were added at a concentration of 25 µg/ml, and hygromycin was used at a concentration of 250 µg/ml. The *E. coli* strain SURE (14) was used as the host for cosmid library preparation, and strain TG1 (12) was used for plasmid construction. *E. coli* S17.1 (39), carrying an integrated RP4 derivative, was used for intergenic conjugation between *E. coli* and *S. albus*. *E. coli* GM2929 (*dam dcm*) was used for the extraction of unmethylated DNA. *E. coli* cells were cultured in Luria broth supplemented with ampicillin (100 µg/ml) when required. pUC19 and M13 vectors (46) were used for cloning and sequencing. pHM11a (28), an *E. coli-Streptomyces* integrative shuttle vector, was used for complementation of the *hrcA* mutation. Cosmid vector SuperCos I (9) was obtained from Stratagene.

**Preparation of the cosmid library.** Chromosomal DNA from *S. albus* was partially digested with *Sau3AI* and ligated into the *Bam*HI site of SuperCos I. The cosmid was packaged by using λ packaging extracts from Stratagene. *E. coli* SURE was infected with the λ particles, and ampicillin-resistant clones were selected. A total of 1,152 colonies were used to inoculate 150 µl of Luria-Bertani broth containing ampicillin in 12 96-well microtiter plates. After overnight growth, glycerol was added to a final concentration of 20%, and the plates were stored at -70°C.

**DNA manipulations.** Degenerate oligonucleotide Dal3 (ACSATCCGSAACG ASATGGCCSASCTSGAG, with S being G or C) based on the HrcA consensus region, TIRNEMADLE, was used to probe the cosmid library. The oligonucleotide was radiolabeled with [ $\gamma$ -<sup>32</sup>P]dATP by using polynucleotide kinase (Pharmacia). The blots were probed overnight by incubation at 57°C with the oligonucleotide. They were then washed with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) at 57°C and autoradiographed. Four colonies that gave a strong signal were analyzed further. In Southern blots probed with radiolabeled Dal3, three cosmids (H1, H2, and H3) generated similar hybridization patterns. In particular, a 1.2-kb *Sall* fragment present in the three preparations hybridized with Dal3. This fragment was isolated from agarose gel and inserted into pUC19. The sequence was determined and showed that this fragment contained the 5' region of a gene similar to *hrcA*. The fourth cosmid, H4, generated a different hybridization pattern. A 450-bp *Sall* fragment which hybridized with Dal3 was cloned and sequenced. It contained a stretch of 22 nucleotides identical to the Dal3 probe, accounting for the hybridization signal. However, the rest of the sequence was very different from that of *hrcA*. The protein encoded by the 450-bp *Sall* fragment had a sequence similar to that of the NuaA protein (SwissProt identification no. P32727) of *B. subtilis*. There were no significant similarities between the sequences of NuaA and HrcA, so we concluded that the signal obtained was due to chance, and cosmid H4 was not analyzed further.

We obtained a complete copy of the *hrcA* gene by isolating a 3.5-kb *Sma*I fragment from cosmid H1 and inserting it into pUC19 cut with *Sma*I, giving pPM3881.

**Determination of the DNA sequence.** The *Eco*RI/*Hind*III fragment of pPM3881 was inserted into the appropriate sites of M13mp18 or M13mp19. DNA was sequenced by the dideoxy chain method of Sanger et al. with the Sequenase kit (version 2.0; U.S. Biochemical Corp.) and <sup>32</sup>S-dATP, the universal M13 primer, and synthetic oligonucleotides (17-mers; Genset).

**Construction of an *hrcA* insertion mutant.** pPM3881 was cut with *Bcl*I, and the 1.8-kb *Bam*HI fragment from pJ39 (41) carrying the *tsr* gene was inserted into the *Bcl*I site of pPM3881 to give pPM3891. pCG3981 was constructed by introducing the *oriT-vioR* cassette (37) into the single *Hind*III site of pPM3891. This cassette contains an origin of transfer and makes it possible to select for viomycin resistance. pPM3981 was transferred by conjugation from *E. coli* to *S. albus* as previously described (26).

**Construction of a *dnaJ2* insertion mutant.** pPM3881 was cut with *Bam*HI and *Alw*NI, and the 980-bp internal fragment of *dnaJ2* was isolated, its ends were blunted, and it was ligated into *Sma*I-digested pUC19, giving pPM3902. The internal *dnaJ2* fragment was removed by digestion with *Kpn*I and *Bam*HI and was inserted into *Kpn*I- and *Bam*HI-digested pPM2208 (formerly called pPM935-del [37]) to give pPM3922. pPM3922 was transferred by conjugation from *E. coli* to *S. albus*, and thio-strepton-resistant clones were selected.

**Secretion of endogenous proteins.** For α-amylase secretion analysis, *S. albus* wild-type and mutant strains were patched on starch-containing plates and halos of starch hydrolysis were detected by iodine staining. For β-lactamase secretion analysis, the strains were patched on ampicillin-containing plates. They were cultured for various periods of time, and the plates were then spread with ampicillin-sensitive *E. coli* bacteria. The size of the halos of *E. coli* growing at the point of contact with the *Streptomyces* patches indicated the level of ampicillin detoxification by secreted β-lactamase.

**Complementation of the *hrcA* mutation.** A full-length *hrcA* gene was obtained by PCR with the oligonucleotides OLC45 (5' CGCATATGCTCAGCGAACGA

AGGCTCGAAG 3') and OLC46 (5' CGGGATCCTTACGACTCCGCCAGG ATCTGTC 3').

The PCR product was cut with *Nde*I and *Bam*HI and ligated into pHM11a digested with *Nde*I and *Bam*HI to give pCG92. pCG92 was transferred by conjugation from *E. coli* to *S. albus*. Exconjugants were selected on the basis of hygromycin resistance.

**Heat shock protein induction in *Streptomyces*.** Total proteins, either [<sup>35</sup>S]methionine-cysteine labeled or unlabeled, were extracted as described previously (16). SDS-polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide) was performed according to the method of Laemmli (23). The proteins were transferred to Immobilon membranes. GroEL proteins were detected by Western blotting with the ECL Western blotting system (Amersham). Mouse antibodies raised against *Mycobacterium leprae* GroEL were provided by B. Gicquel (Institut Pasteur).

**RNA analysis.** *S. albus* total RNA was extracted, and Northern blotting was carried out as previously described (38). Highly stringent conditions were used for hybridization, and the blots were washed with 0.5× SSC-0.1% SDS at 65°C. The probes used were *S. albus groES*, *groEL2* (25), and *hrcA* (this work).

The transcription start site upstream from the *hrcA-dnaJ2* operon was determined by primer extension. The *hrcA*-specific oligonucleotide OLC47 (5' GCG GTACCTTGTCGGTTCGGGATCGGCC 3'), radiolabeled with [ $\gamma$ -<sup>32</sup>P]dATP by using polynucleotide kinase, was used with RNA preparations from heat-shocked and untreated *S. albus* cultures. Avian myeloblastosis virus (AMV) reverse transcriptase was used according to the manufacturer's recommendations (Pharmacia). The samples were subjected to electrophoresis in a 5% polyacrylamide-8 M urea gel.

**Nucleotide sequence accession number.** The nucleotide sequence of *S. albus hrcA* has been deposited in GenBank (accession no. AF025656).

## RESULTS

**Cloning of the *hrcA* gene of *S. albus*.** The CIRCE operator sequences are highly conserved among bacterial species, but the sequences of HrcA repressors share few similarities. This makes it difficult to design a nucleotide probe for *hrcA*, even if the *Streptomyces* codon bias is used. *Streptomyces* has a high G+C content, and it tends to favor codons with a G or a C in the third position. However, we mostly used highly degenerate probes. Various oligonucleotides were designed based on the available HrcA sequences. Oligonucleotide Dal3 is based on the peptide TIRN(EDY)MA(DAQV)LE, a consensus sequence derived from the HrcA proteins of *B. subtilis* (44), *Staphylococcus aureus* (31), *Clostridium acetobutylicum* (30), *Mycoplasma genitalium* (10), and *Chlamydia trachomatis* (40), and on *ygr*, a sequence encoding an HrcA-like protein, discovered during the *M. leprae* sequencing project (cosmid B1937; K. Robison, GenBank). Oligonucleotide Dal3 hybridized strongly to four clones of the *S. albus* cosmid library. Three of the clones were found to have sequences similar to that of *hrcA*, whereas the fourth was a false positive.

**Nucleotide sequence of *S. albus hrcA* and *dnaJ2*.** The nucleotide sequence of *hrcA* and its downstream and upstream regions (2,521 bp) was determined by using plasmid pPM3881 and subclones in M13 vectors. The sequence was aligned with those of HrcA proteins, and a GTG translation initiation codon was identified, preceded by a GGAGG ribosome-binding site. The *hrcA* gene encodes a protein of 336 amino acids, with a molecular mass of 36.9 kDa and a sequence 26% identical to that of the *B. subtilis* HrcA. The deduced peptide sequence contained the sequence TVRNDMAALE rather than the consensus TIRN(EDY)MA(DAQV)LE.

There was also a "dnaJ-like" gene immediately downstream from *hrcA*. As there was already a *dnaJ* gene in the *dnaK* operon of *Streptomyces*, we called this gene *dnaJ2*. It encodes a 379-amino-acid polypeptide, with a predicted molecular mass of 40.6 kDa. A sequence with dyad symmetry downstream from *dnaJ2* may be a transcriptional terminator. The *hrcA* gene is preceded by a canonical CIRCE sequence TTGGCACTC 9N GAGTGCCAG 78 bp upstream from the putative GTG translation start site of *hrcA*.

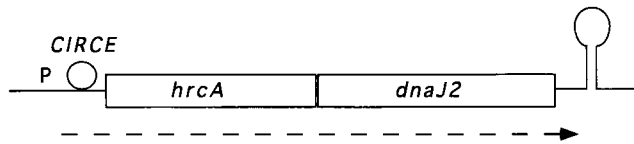


FIG. 1. Schematic representation of the *hrcA-dnaJ2* operon. The promoter (P), CIRCE motif (circle), and putative transcription terminator (hairpin) are indicated. Polycistronic mRNA is represented by a dashed arrow.

**Transcription of the *hrcA-dnaJ2* operon.** The transcription of *hrcA* was investigated by Northern blotting with an internal *hrcA* probe. A 2.4-kb transcript was detected in RNA extracted from heat-shocked cultures (data not shown). An mRNA of this size was predicted from the sequence of an *hrcA-dnaJ2* polycistronic transcript, starting near the CIRCE element and ending at the putative transcription terminator. This result also suggests that the transcription of the *hrcA-dnaJ2* operon is heat induced, and this was confirmed by a primer extension experiment, as described below. The arrangement of the genes of this new operon is shown in Fig. 1.

**Determination of the transcription initiation site.** We determined the transcription start site of the *hrcA-dnaJ2* operon by primer extension analysis with oligonucleotide OLC47 on RNA isolated from cultures grown at 30°C or heat shocked at 41°C for 20 min (Fig. 2).

A single transcription start site was identified 87 bp upstream from the *hrcA* translation initiation codon and 6 bp upstream from the CIRCE element. The -35 and -10 hexamers were aligned, and the following similarities were found between the *Streptomyces* vegetative promoter consensus and the *hrcA* promoter: TTGACPu17-18ntAgPuPuT for the vegetative promoter and TTGCGC19ntACACT for the *hrcA-dnaJ2* promoter, where nt is nucleotides. The space between the -10 and -35 hexamer motifs is exceptionally long, with 19 bp rather than 17 or 18 bp. The -35 hexamer of the *hrcA* promoter (boldface) is located within a palindromic sequence (underlined), GGTGTGCCCGGCCTTGGCGCTCGGCACACC, which may be involved in the regulation of expression of this gene (see Discussion).

**Disruption of the *hrcA* gene and resulting phenotype.** We disrupted the *hrcA* gene of *S. albus* by using a technique involving the conjugative transfer from *E. coli* to *Streptomyces* of a plasmid unable to replicate in *Streptomyces*. Conjugation between *E. coli* S17-1 containing pPM3891 and *S. albus* resulted in thiostrepton-resistant exconjugants, which were screened for viomycin sensitivity. The frequency of viomycin-sensitive, thiostrepton-resistant clones (Tsr<sup>r</sup> Vio<sup>s</sup>) was about 6%. The *hrcA* gene of these mutants was assumed to be disrupted by a *tsr* insertion following a double-crossover event at the *hrcA* locus. The elimination of vector DNA and integration of *tsr* into the genome was demonstrated by Southern blotting (data not shown).

Growth of the *hrcA* mutant was not impaired at 30°C on solid rich medium. The formation of the aerial mycelium and sporulation, in particular, were not prevented at 30 or 37°C. However, the mutant grew more slowly at 37°C than the wild type did.

GroEL facilitates the export of some proteins from *E. coli* (24, 32). Endogenous amylase and  $\beta$ -lactamase secretion was assessed by plate assays, and no obvious effect of GroEL accumulation on the export of these two proteins from the *hrcA* mutant was observed (results not shown).

**Analysis of an *hrcA* null mutation on the expression of the principal *hsp* genes.** The levels of GroES and GroEL proteins in the *hrcA* mutant were assessed by SDS-PAGE (Fig. 3).

There were large amounts of a 60- and an 11-kDa protein in the protein extract of the mutant. The overproduced proteins were recognized by anti-GroEL (Fig. 4) and anti-GroES antibodies (data not shown). Anti-GroEL antibodies detected several minor bands for the mutant, which were not detected for the wild-type control. These bands are probably GroEL degradation products. A high level of proteolysis was observed for various proteins in *E. coli* cells overproducing GroEL or GroEL and GroES (21).

We scanned the Coomassie blue-stained gel and found that the 60-kDa protein accounted for 19% of total protein in the *hrcA* mutant and the 11-kDa protein accounted for 5.5%. This provides enough GroES versus GroEL for the formation of the chaperonin machinery. Indeed, in the classical asymmetric GroEL-GroES chaperonin complex (6), 14 subunits of GroEL (56 kDa) interact with 7 subunits of GroES (11 kDa). The predicted protein ratio (wt/wt) between GroEL and GroES in this complex is around 10. The protein ratio is around 5 for a symmetric 14-GroEL-14-GroES complex.

Northern blotting with a *groES* probe showed that the *groES* and *groES-groEL1* transcripts accumulated in the *hrcA* mutant at 30°C (Fig. 5). These transcripts did not accumulate in a control in which the mutation was complemented by pCG92, which contains a functional copy of *hrcA*. The blot was probed

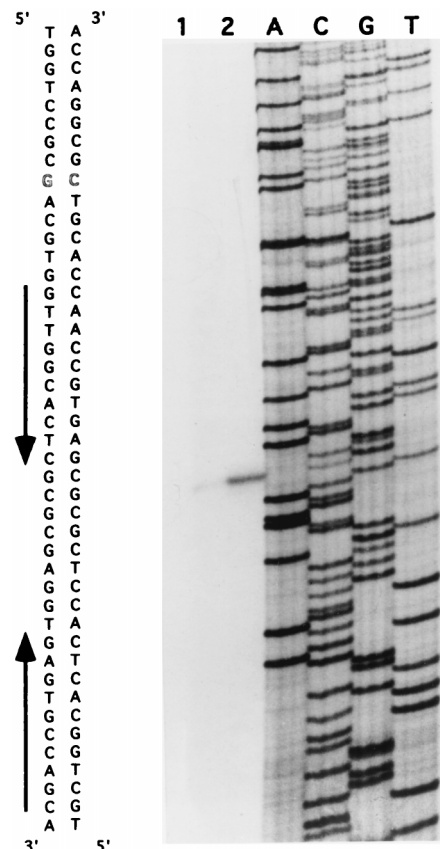


FIG. 2. Determination of the transcription start site of the *hrcA-dnaJ2* operon. Primer extension mapping with the AMV reverse transcriptase was carried out with 5'-labeled oligonucleotide OLC47 and RNA from cultures grown at 30°C (lane 1) or subjected to heat shock at 41°C for 20 min (lane 2). The sizes of the fragments were determined by electrophoresis and comparison with sequencing ladders (lanes A, C, G, and T) generated with OLC47 on single-stranded DNA (a subclone from pPM3881 in M13mp18). The relevant DNA sequence is indicated, and the transcription start site is shown by outlined characters. The converging arrows indicate the CIRCE sequence.

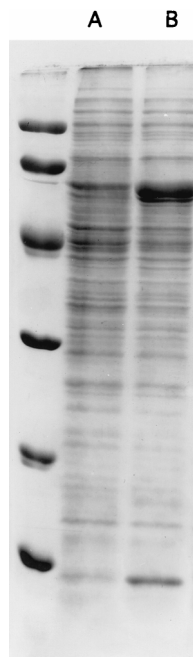


FIG. 3. Accumulation of GroEL and GroES in the *hrcA* mutant. *S. albus* wild-type (lane A) and *hrcA* null-mutant (lane B) bacteria were grown at 30°C for 18 h. Protein extracts were prepared and subjected to SDS-PAGE (15% polyacrylamide). The gel was stained with Coomassie blue R250. Molecular mass markers (top to bottom): 94, 67, 43, 30, 20.1, and 14.4 kDa.

with a *groEL2*-specific probe, and *groEL2* transcripts were found to accumulate similarly in the noncomplemented *hrcA* mutant (data not shown).

The pattern of expression of the principal, CIRCE-independent *hsp* genes (*hsp94*, *dnaK*, and *hsp18*) was not affected by the *hrcA* mutation. Labeling experiments with [<sup>35</sup>S]methionine-cysteine showed that these genes underwent normal thermoinduction (Fig. 6).

**Complementation of the *hrcA* null mutation.** Insertion of the *tsr* resistance gene is likely to have a polar effect on the expression of *dnaJ2*, the second gene of the operon. We investigated the involvement of *hrcA* and *dnaJ2* in generating the *hrcA* null

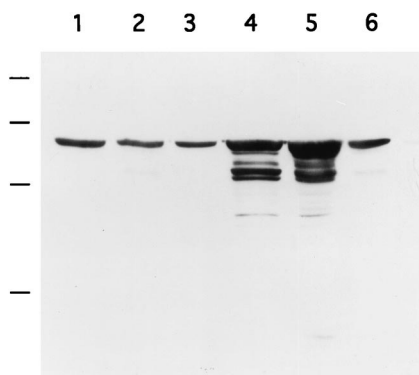


FIG. 4. Immunological detection of GroEL. Cultures of the *S. albus* wild type (lane 1), wild type with vector pHM11a (lane 2), wild type with pCG92 (lane 3), *hrcA* null mutant (lane 4), *hrcA* mutant with pHM11a (lane 5), and *hrcA* mutant complemented by pCG92 (lane 6) were grown at 30°C. Total cell protein extracts were prepared, and equal amounts of protein were applied to each lane. The immunoblot was probed with antibodies raised against *M. leprae* GroEL. Molecular mass markers (the bars adjacent to lane 1; top to bottom): 94, 67, 43, and 30 kDa.

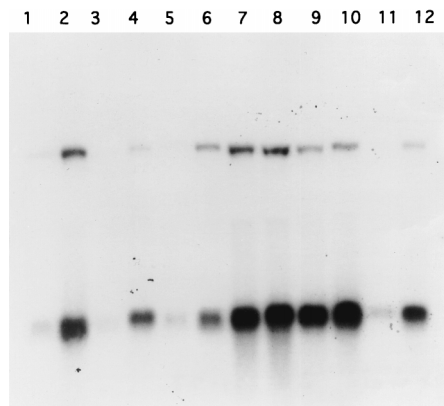


FIG. 5. Northern blot analysis of the *groES* and *groESL1* transcripts. Total RNA was extracted from cultures of the *S. albus* wild type (lanes 1 and 2), wild type with vector pHM11a (lanes 3 and 4), wild type with pCG92 (lanes 5 and 6), *hrcA* null mutant (lanes 7 and 8), *hrcA* mutant with vector pHM11a (lanes 9 and 10), and *hrcA* mutant complemented by pCG92 (lanes 11 and 12). The cultures were grown at 30°C (lanes 1, 3, 5, 7, 9, and 11) or shifted to 41°C for 20 min (lanes 2, 4, 6, 8, 10, and 12). RNAs were hybridized with a *groES*-specific probe which detects both the monocistronic *groES* and the polycistronic *groES-groESL1* products of the *groESL1* operon.

phenotype by complementing the mutation with pCG92 in *trans*. pCG92 contains a copy of *hrcA* under the control of the constitutive *erm* promoter from the erythromycin resistance gene of *Saccharopolyspora erythraea*.

The patterns of *groESL1* and *groEL2* expression in the *hrcA* mutant complemented with pCG92 were similar to those of the wild-type strain at 30°C and after heat shock. This shows that HrcA represses the transcription of *groESL1* and *groEL2*. The pHM11a vector alone has no effect on *groESL* expression. The

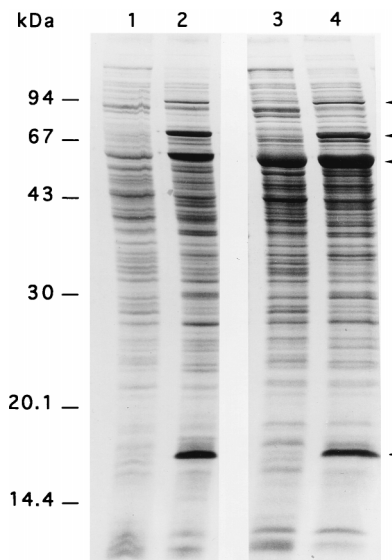


FIG. 6. Effect of the *hrcA* mutation on the kinetics of HSP synthesis after temperature shift in *S. albus*. Cultures (20 h after inoculation) of the *S. albus* wild type (lanes 1 and 2) or *hrcA* null mutant (lanes 3 and 4) growing at 30°C were divided into 1-ml aliquots, which were cultured at 30°C (lanes 1 and 3) or 41°C (lanes 2 and 4). Five minutes later, [<sup>35</sup>S]methionine-cysteine was added, and the cultures were labeled by incubation for 20 min. Total protein extracts were prepared and subjected to SDS-PAGE, and the gel was dried and subjected to autoradiography. HSP94, HSP70 (DnaK), HSP56-58 (GroEL), and HSP18 (top to bottom) are indicated by arrows. GroES does not contain methionine or cysteine and therefore is not detected.

*hrcA* gene in pCG92 was constitutively expressed under the control of the *erm* promoter, so regulation of *groESL1* and *groEL2* transcription was not directly linked to the level of *hrcA* transcription. High-level constitutive expression of *hrcA* did not prevent *groES-groEL1* and *groEL2* thermoinduction (Fig. 4 and 5). Therefore, regulation probably depends on the modulation of HrcA activity (see Discussion).

**Disruption of *dnaJ2*.** Our attempts to disrupt *dnaJ2*, the second gene of the operon, were at first unsuccessful. We used a procedure similar to that used to disrupt *hrcA* but were unable to select the double-crossover event leading to insertion of a cassette into *dnaJ2*. We then used an internal *dnaJ2* fragment to screen for the insertion of the plasmid in a single-recombination event (one crossover), which is more readily selected (37). Clones with disruption of *dnaJ2* were selected, and the integration of the plasmid into *dnaJ2* was confirmed by Southern blotting. The growth of the *dnaJ2* mutant on NE plates was similar to that of the wild type. The pattern of HSPs in SDS-PAGE was also similar to that of the wild-type strain (data not shown). However, in this mutant, of the two deletion-containing copies of *dnaJ2* obtained by this procedure, one had a deletion of only 45 bp at its 3' end. It is not clear whether this deletion is sufficient to prevent the formation of an active gene product.

## DISCUSSION

We identified *hrcA*, which encodes the repressor of the *groESL1* and *groEL2* genes in *S. albus*. In an earlier work we reported that *groEL1* was regulated transcriptionally and by a thermodependent posttranscriptional regulation (38). Here we show that disruption of the repressor led to *groE* transcript and protein accumulation at low temperatures, showing that post-transcriptional regulation, by whatever mechanism, did not prevent translation of the accumulated transcripts in the absence of HrcA. Protein synthesis was analyzed by [<sup>35</sup>S]methionine-cysteine labeling of *hrcA* mutant cultures grown at 30°C or under heat shock conditions. Temperature had no significant effect on GroEL biosynthesis in the mutant.

The expression of the *hrcA* gene was thermoinducible. There is a CIRCE sequence at the 5' end of the *hrcA-dnaJ2* transcript, so HrcA probably negatively autoregulates its own synthesis by binding to CIRCE. *hrcA* transcription may also be subject to positive regulation. The -35 hexamer of the *hrcA* promoter is located in a palindromic sequence, and the space between the -10 and -35 hexamer motifs is longer than usual: 19 bp rather than 17 or 18 bp. The situation in the mercury resistance gene cluster is similar. In the *merT* promoter, a 19-bp space between the -10 and -35 hexamers puts these two elements out of alignment with the RNA polymerase regions with which they must interact. Binding of the MerR regulator to a nearby palindromic sequence induces a 33° unwinding, making it possible for the hexamers to align in such a way that they function (18). A similar mechanism has been suggested for the thioestrepton-inducible promoter *tipAp* in *Streptomyces* (19). Positive regulation by twisting the promoter may also apply to *hrcA*.

*hrcA* was associated with a second copy of *dnaJ*. The presence of several *dnaJ* genes is not unusual. There is a second copy of *dnaJ*, *cbpA*, in *E. coli* (42), and there are three *dnaJ*-like sequences in the small *M. genitalium* genome (10). The presence of two *dnaJ* genes suggests that the encoded proteins may have different physiological functions, either alone or in association with DnaK. The *dnaJ259* mutation in *E. coli*, resulting from an H33D change in the HPD tripeptide, prevents interaction with DnaK (43). The HPD motif is present in both DnaJ

and DnaJ2, so both may interact with DnaK. DnaJ2 has the canonical organization of all members of the DnaJ family: an amino-terminal J domain, then a glycine-rich domain, zinc fingers composed of repeats of the CXXCXGXG motif, and a low-homology carboxy-terminal region.

The slight inhibition of the growth of the *hrcA* mutant at 37°C was unexpected because accumulation of GroEL increases *E. coli* survival at high temperatures (22).

The secretion of heterologous proteins in the *hrcA* mutant will be analyzed in detail as part of another study.

GroESL is the major modulator of the CIRCE heat shock regulon in *B. subtilis* (27). The interaction of GroEL and HrcA in this bacterium controls the availability of the repressor form of HrcA. It will be of interest to determine whether one or both of the *Streptomyces* GroEL proteins interact with HrcA in a similar way. Five *groEL* genes have been characterized in *Bradyrhizobium japonicum*. The *groESL4* gene is regulated by CIRCE, whereas *groESL1* depends on sigma 32 factor for transcription. Knockout mutations in *groEL4* resulted in higher levels of *groESL4* transcription. This suggests that CIRCE repression depends on the cellular level of GroEL4 (1). HrcA activity may well be regulated by another component in *Streptomyces*. Indeed, we have shown that overexpression of *hrcA*, either by thermoinduction or by insertion of the gene in an expression vector, does not lead to repression of CIRCE-regulated genes. In particular, the thermoinduction of *hrcA*, which is overexpressed at the point at which it stops acting as a repressor, suggests a situation similar to that described for *B. subtilis*, in which the modulator of HrcA activity is titrated under heat shock conditions (27).

This work provides no evidence for a correlation between the *Streptomyces* cell cycle and *groEL* regulation. Instead, it complements previous work, in which *hspR* and *orfY*, two other regulators of the principal chaperones of *Streptomyces*, were disrupted or overexpressed (13, 36). In each case, changing the amount of the regulator had no direct effect on the formation of the aerial mycelium or sporulation. Here we show that this is also the case for *hrcA*. Taken together, these results indicate that the regulatory systems of the main HSPs, GroEL, DnaK, and Hsp18, are not directly involved in *Streptomyces* development.

## ACKNOWLEDGMENTS

We thank P. Servant, V. De Crecy, and W. Schumann for their constant interest in this work. We are grateful to J. Rauzier for his help in sequencing the various *hrcA* candidates that we obtained. We thank H. Motamedi for supplying pHM11a and B. Gicquel for anti-GroEL and anti-GroES antibodies.

This work was supported by the Pasteur Institute and the CNRS.

## REFERENCES

- Babst, M., H. Hennecke, and H. M. Fischer. 1996. Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. *Mol. Microbiol.* **19**:827-839.
- Bucca, G., G. Ferina, A. M. Puglia, and C. P. Smith. 1995. The *dnaK* operon of *Streptomyces coelicolor* encodes a novel heat-shock protein which binds to the promoter region of the operon. *Mol. Microbiol.* **17**:663-674.
- Bukau, B. 1993. Regulation of the *Escherichia coli* heat-shock response. *Mol. Microbiol.* **9**:671-680.
- Chater, K. F. 1993. Genetics of differentiation in *Streptomyces*. *Annu. Rev. Microbiol.* **47**:685-713.
- Chater, K. F., and L. C. Wilde. 1980. *Streptomyces albus* G mutants defective in the *SalGI* restriction-modification system. *J. Gen. Microbiol.* **116**:323-334.
- Chen, S., A. M. Roseman, A. S. Hunter, S. P. Wood, S. G. Burston, N. A. Ranson, A. R. Clarke, and H. R. Saibil. 1994. Location of a folding protein and shape changes in GroEL-GroES complexes imaged by cryo-electron microscopy. *Nature* **371**:261-264.
- Duchêne, A.-M., H. M. Kieser, D. A. Hopwood, C. J. Thompson, and P. Mazodier. 1994. Characterization of two *groEL* genes in *Streptomyces coelicolor* A3(2). *Gene* **144**:97-101.

8. Duchêne, A.-M., C. J. Thompson, and P. Mazodier. 1994. Transcriptional analysis of *groEL* genes in *Streptomyces coelicolor* A3(2). *Mol. Gen. Genet.* **245**:61–68.
9. Evans, G. A., K. Lewis, and B. E. Rothenberg. 1989. High efficiency vectors for cosmid microcloning and genomic analysis. *Gene* **79**:9–20.
10. Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, et al. 1995. The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**:397–403.
11. Georgopoulos, C., K. Liberek, M. Zylicz, and D. Ang. 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response, p. 209–249. In R. I. Morimoto, A. Tissières, and C. Georgopoulos (ed.), *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
12. Gibson, T. J. 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. Cambridge University, Cambridge, England.
13. Grandvalet, C., P. Servant, and P. Mazodier. 1997. Disruption of *hspR*, the repressor gene of the *dnaK* operon in *Streptomyces albus* G. *Mol. Microbiol.* **23**:77–84.
14. Greener, A. 1990. *E. coli* SURE clones “unclonable” DNA. *Strategies* **3**:5–6.
15. Guglielmi, G., A.-M. Duchêne, C. Thompson, and P. Mazodier. 1993. Transcriptional analysis of two different *Streptomyces albus* *groEL*-like genes, p. 17–24. In R. H. Baltz, G. D. Hegeman, and P. L. Skatrud (ed.), *Industrial microorganisms: basic and applied molecular genetics*. American Society for Microbiology, Washington, D.C.
16. Guglielmi, G., P. Mazodier, C. J. Thompson, and J. Davies. 1991. A survey of the heat shock response in four *Streptomyces* species reveals two *groEL*-like genes and three GroEL-like proteins in *Streptomyces albus*. *J. Bacteriol.* **173**:7374–7381.
17. Hecker, M., W. Schumann, and U. Volker. 1996. Heat-shock and general stress response in *Bacillus subtilis*. *Mol. Microbiol.* **19**:417–428.
18. Heltzel, A., I. W. Lee, P. A. Totis, and A. O. Summers. 1990. Activator-dependent preinduction binding of sigma-70 RNA polymerase at the metal-regulated *mer* promoter. *Biochemistry* **29**:9572–9584.
19. Holmes, D. J., J. L. Caso, and C. J. Thompson. 1993. Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. *EMBO J.* **12**:3183–3191.
20. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf. 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
21. Kandror, O., L. Busconi, M. Sherman, and A. L. Goldberg. 1994. Rapid degradation of an abnormal protein in *Escherichia coli* involves the chaperones GroEL and GroES. *J. Biol. Chem.* **269**:23575–23582.
22. Kandror, O., and A. L. Goldberg. 1997. Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. *Proc. Natl. Acad. Sci. USA* **94**:4978–4981.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
24. Laminet, A. A., T. Ziegelhoffer, C. Georgopoulos, and A. Plückthun. 1990. The *Escherichia coli* heat shock proteins GroEL and GroES modulate the folding of the  $\beta$ -lactamase precursor. *EMBO J.* **9**:2315–2319.
25. Mazodier, P., G. Guglielmi, J. Davies, and C. J. Thompson. 1991. Characterization of the *groEL*-like genes in *Streptomyces albus*. *J. Bacteriol.* **173**:7382–7386.
26. Mazodier, P., R. Petter, and C. Thompson. 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. *J. Bacteriol.* **171**:3583–3585.
27. Mogk, A., G. Homuth, C. Scholz, L. Kim, F. X. Schmid, and W. Schumann. 1997. The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. *EMBO J.* **16**:4579–4590.
28. Motamedi, H., A. Shafiee, and S. J. Cai. 1995. Integrative vectors for heterologous gene expression in *Streptomyces* spp. *Gene* **160**:25–31.
29. Murakami, T., H. Anzai, S. Imai, A. Satoh, K. Nagaoka, and C. Thompson. 1986. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning of the gene cluster. *Mol. Gen. Genet.* **205**:42–50.
30. Narberhaus, F., K. Giebeler, and H. Bahl. 1992. Molecular characterization of the *dnaK* gene region of *Clostridium acetobutylicum*, including *grpE*, *dnaJ*, and a new heat shock gene. *J. Bacteriol.* **174**:3290–3299.
31. Ohta, T., K. Saito, M. Kuroda, K. Honda, H. Hirata, and H. Hayashi. 1994. Molecular cloning of two new heat shock genes related to the *hsp70* genes in *Staphylococcus aureus*. *J. Bacteriol.* **176**:4779–4783.
32. Phillips, G. J., and T. J. Silhavy. 1990. Heat-shock proteins DnaK and GroEL facilitate export of LacZ hybrid protein in *E. coli*. *Nature* **344**:882–884.
33. Puglia, A. M., J. Vohradsky, and C. J. Thompson. 1995. Developmental control of the heat shock stress regulon in *Streptomyces coelicolor*. *Mol. Microbiol.* **17**:737–746.
34. Redenbach, M., H. M. Kieser, D. Denapate, A. Eichner, J. Cullum, H. Kinashi, and D. A. Hopwood. 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol. Microbiol.* **21**:77–96.
35. Schulz, A., and W. Schumann. 1996. *hrcA*, the first gene of the *Bacillus subtilis* *dnaK* operon encodes a negative regulator of class I heat shock genes. *J. Bacteriol.* **178**:1088–1093.
36. Servant, P., and P. Mazodier. 1996. Heat induction of *hsp18* gene expression in *Streptomyces albus* G: transcriptional and posttranscriptional regulation. *J. Bacteriol.* **178**:7031–7036.
37. Servant, P., C. Thompson, and P. Mazodier. 1993. Use of new *Escherichia coli*/*Streptomyces* conjugative vectors to probe the functions of the two *groEL*-like genes of *Streptomyces albus* G by gene disruption. *Gene* **134**:25–32.
38. Servant, P., C. Thompson, and P. Mazodier. 1994. Post-transcriptional regulation of the *groEL1* gene of *Streptomyces albus*. *Mol. Microbiol.* **12**:423–432.
39. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784–791.
40. Tan, M., B. Wong, and J. N. Engel. 1996. Transcriptional organization and regulation of the *dnaK* and *groE* operons of *Chlamydia trachomatis*. *J. Bacteriol.* **178**:6983–6990.
41. Thompson, C. J., J. M. Ward, and D. A. Hopwood. 1980. DNA cloning in *Streptomyces*: resistance genes from antibiotic producing species. *Nature* **286**:525–527.
42. Ueguchi, C., M. Kakeda, H. Yamada, and T. Mizuno. 1994. An analogue of the DnaJ molecular chaperone in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **91**:1054–1058.
43. Wall, D., M. Zylicz, and C. Georgopoulos. 1994. The NH<sub>2</sub>-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication. *J. Biol. Chem.* **269**:5446–5451.
44. Wetzstein, M., U. Völker, J. Dedio, S. Löbau, U. Zuber, M. Schiesswohl, C. Herget, M. Hecker, and W. Schumann. 1992. Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. *J. Bacteriol.* **174**:3300–3310.
45. Wild, J., E. Altman, T. Yura, and C. A. Gross. 1992. DnaK and DnaJ heat shock proteins participate in protein export in *Escherichia coli*. *Genes Dev.* **6**:1165–1172.
46. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
47. Yuan, G., and S. L. Wong. 1995. Isolation and characterization of *Bacillus subtilis* *groE* regulatory mutants: evidence for *orf39* in the *dnaK* operon as a repressor gene in regulating the expression of both *groE* and *dnaK*. *J. Bacteriol.* **177**:6462–6468.
48. Yura, T., H. Nagai, and H. Mori. 1993. Regulation of the heat-shock response in bacteria. *Annu. Rev. Microbiol.* **47**:321–350.
49. Zuber, U., and W. Schumann. 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis*. *J. Bacteriol.* **176**:1359–1363.