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hrcA, the First Gene of the *Bacillus subtilis dnaK* Operon Encodes a Negative Regulator of Class I Heat Shock Genes

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Whereas in *Escherichia coli* only one heat shock regulon is transiently induced by mild heat stress, for *Bacillus subtilis* three classes of heat shock genes regulated by different mechanisms have been described. Regulation of class I heat shock genes (*dnaK* and *groE* operons) involves an inverted repeat (CIRCE element) which most probably serves as an operator for a repressor. Here, we report on the analyses of an *hrcA* null mutant (Δ*hrcA*), in which *hrcA*, the first gene of the *dnaK* operon, was deleted from the *B. subtilis* chromosome. This strain was perfectly viable at low and high temperatures. Transcriptional analysis of the deletion mutant revealed a high level of constitutive expression of both the *dnaK* and *groE* operons even at a low temperature. A further increase in the amount of *groE* transcript was observed after temperature upshift, suggesting a second induction mechanism for this operon. Overproduction of HrcA protein from a second copy of *hrcA* derived from a plasmid (p*hrcA*$^+$) in *B. subtilis* wild-type and Δ*hrcA* strains prevented heat shock induction of the *dnaK* and *groE* operons at the level of transcription almost completely and strongly reduced the amounts of mRNA at a low temperature as well. Whereas the wild-type strain needed 4 h to resume growth after temperature upshift, the Δ*hrcA* strain stopped growth only for about 1 h. Overproduction of HrcA protein prior to a heat shock almost completely prevented growth at a high temperature. These data clearly demonstrate that the *hrcA* product serves as a negative regulator of class I heat shock genes.

The heat shock response is an important homeostatic mechanism that enables cells from animals, plants, and bacteria to survive a variety of environmental stresses (21, 22). It is characterized by the transiently increased synthesis of a number of proteins, which are called heat shock proteins (HSPs). The strong evolutionary conservation of the heat shock response argues that this response is beneficial for many kinds of cells. HSPs have essential roles in the synthesis, transport, and folding of proteins and are often referred to as molecular chaperones (9). In prokaryotes, the major HSPs are encoded by single genes expressed constitutively at all temperatures. Following a temperature upshift, the rates of expression of these genes abruptly accelerate. After about 8 min, the rates of synthesis of the HSPs are turned down. In *Escherichia coli*, the heat shock response is positively regulated by the alternate sigma factor σ^{32} and is negatively regulated by the products of the heat shock genes *dnaK*, *dnaJ*, and *grpE* (for recent reviews, see references 5 and 39).

In contrast to *E. coli*, *Bacillus subtilis* contains three classes of heat shock genes which are turned on by mild heat stress (12). Class I heat shock genes, as exemplified by the *dnaK* and the *groE* operons, are expressed from the vegetative promoter P_A (6), and their expression involves a *cis*-active inverted repeat called CIRCE (41). We suggested that class I heat shock genes are negatively regulated by a repressor interacting with the CIRCE element (31). Class II is composed of about 40 different genes (11), and these genes are regulated by the alternate sigma factor σ^B. Class III heat shock genes are expressed from vegetative promoters P_A, and additional elements are still uncharacterized. Here, the genes *lon* (24), *clpC* (17), and *ftsH* (7) have been identified so far.

The *dnaK* operon of *B. subtilis* starts with an open reading frame (ORF) formerly called *orf39*. Recently, R. Roberts identified the *orf39* homolog of *Caulobacter crescentus*, inactivated that gene, and was able to show that it acts as a negative regulator. Therefore, he suggested naming this ORF *hrcA* for heat regulation at CIRCE (25). The *B. subtilis hrcA* gene encodes a 39-kDa protein and is followed by the three genes *grpE*, *dnaK*, and *dnaJ*. Recently, we reported the isolation of an *hrcA* insertion mutant (31). In that mutant, the unlinked *groE* operon was constitutively expressed at a high rate already at a low temperature. This was not the case in a *dnaK* deletion/insertion mutant, suggesting that either *hrcA* or *grpE* (or both) acts as a negative regulator. To address this question more specifically, an *hrcA* deletion mutant was isolated and analyzed. In addition, *hrcA* was fused to a strong inducible promoter, thereby allowing its controllable expression independently of the growth temperature. Our results clearly demonstrate that *hrcA* encodes a negative regulator of class I heat shock genes.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *E. coli* strain DH5 (10) and the *B. subtilis* strain 1012 (26) were used throughout all of these experiments. BT01 (*hrcA*::*cat*) and BT02 (*dnaK*::*cat*) have been described previously (31). Bacteria were routinely grown aerobically at 30°C in Luria broth (LB). Spizizen minimal medium (SMM) has been described elsewhere (33). Ampicillin, chloramphenicol, and kanamycin were added at concentrations of 50, 5, and 10 μg ml^{-1}, respectively. Plasmids pNEXT33A (15) pBTZ01 (31), pMWD25 (36), pREP9 (18), and p602/22 (18) have been previously described. Plasmid pBTZ02 corresponds to pBTZ01 with a *Not*I linker at the unique *Hin*dIII site. Plasmid pBTZ03 contains the 4.3-kb *Sca*I-*Nco*I fragment from pBTZ02 and the 3.5-kb *Sca*I-*Nco*I fragment from pMWD25.

DNA manipulations and analysis. Standard methods were used for DNA isolation, restriction endonuclease analyses, and ligation (27). Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from New England Biolabs, Stratagene, and Boehringer Mannheim and were used as recommended by the suppliers. Digoxigenin (DIG)-[11]-ddUTP and the DIG detection kit were purchased from Boehringer Mannheim. Nucleotide sequences were determined by the dideoxy nucleotide chain termination method (28). DNA amplifications were conducted in 50-μl reaction mixtures, with the PCR kit as specified (Perkin-Elmer Corp.). The Sequenase DNA sequencing kit was from U.S. Biochemical Corp. Primers were purchased from MWG-BIOTECH, Ebersberg, Germany.

Measurement of the relative amounts of DnaK and GroEL proteins. The relative amounts of DnaK and GroEL proteins within two different *B. subtilis* strains were determined by rocket immunoelectrophoresis (20).

Construction of an *hrcA* deletion mutant. To delete the largest part of the *hrcA* gene from the *B. subtilis* chromosome, we took advantage of two restriction enzyme recognition sites, one situated right at the beginning of the gene (*Hpa*I) and the other near its 3′ end (*Eco*RV) (36). Plasmid pBTZ01 was cut with both endonucleases, and the larger of the two fragments was religated. The resulting deletion plasmid (pAS39Del) was then used to transform the *B. subtilis* strain BT01 which carries an insertion of a *cat* cassette within the *Hpa*I site of *hrcA* (31) together with pNEXT33A according to the procedure described by Itaya et al. (15). Kanamycin-resistant colonies were screened for chloramphenicol sensitivity, and candidates were tested for the replacement of the *cat* cassette by the deletion. One strain (the Δ*hrcA* strain) was kept for further studies. In that strain, two thirds of the *hrcA* gene are deleted, and the truncated gene allows synthesis of a hypothetical peptide of 11 amino acid residues in which only the first two are authentic, while the remaining nine result from a frameshift. With extracts from the Δ*hrcA* strain, no protein band corresponding to HrcA was detectable in immunoblots (data not shown). Furthermore, in Southern blot experiments, with ON1 as probe, the 3.2-kb *Eag*I fragment of wild-type *B. subtilis* was reduced to 2.5 kb in the Δ*hrcA* strain (data not shown).

Analyses of transcription. Isolation of total RNA and slot blot analyses were performed as described previously (36). The following synthetic oligonucleotides complementary to the noncoding strands were used as hybridization probes: ON1 (5′-GCAGCGACCGAACTCGGG-3′; 3′ end of *hrcA*, outside the *Hpa*I-*Eco*RV deletion), ON2 (5′-CTTGCTCTTCTGTTTCG-3′; internal part of *grpE*), ON3 (5′-GCAGAATCCGGCAACAC-3′; internal part of *groES*), and ON4 (5′-GTTGTACCGTCACCGGC-3′ internal part of *groEL*). These oligonucleotides were labeled at their 5′ termini with DIG-[11]-ddUTP as described previously (40).

Construction of a plasmid overproducing HrcA protein. To obtain regulated expression of *hrcA*, this gene was fused to an isopropyl-β-D-thiogalactopyranoside (IPTG)-promoter. First, *hrcA* was amplified by PCR with pBTZ03 as the template. The resulting amplicons were first cloned into p602/22 to allow verification of the correct DNA sequence. A copy of *hrcA* with no mutation was then subcloned as a *Bam*HI fragment into the *E. coli-B. subtilis* shuttle expression vector pREP9, resulting in p*hrcA*$^+$. Two of the *hrcA* amplicons turned out to have single point mutations resulting in an Ile-to-Thr replacement at position 9 in one case (p*hrcA*I9T) and a Glu-to-Gly replacement at position 68 in the second case (p*hrcA*E68G).

RESULTS

An *hrcA* deletion mutant exhibits a high level of constitutive expression of the *dnaK* and the *groE* operons already at a low temperature. The *dnaK* operon consists of at least four genes (see Discussion) in the order *hrcA-grpE-dnaK-dnaJ* (36). Recently, we reported that an *hrcA* insertion mutant (BT01) led to a high constitutive expression of the *groE* operon, whereas no *dnaK* operon-specific transcript was detectable in this strain because of the *cat* insertion at the beginning of *hrcA* (31). In contrast, expression of the *groE* operon and also that of the *dnaK* operon were only slightly increased, if at all, at low temperature in a *dnaK* deletion/insertion mutant (BT02) (31). We inferred from these results that either *hrcA* or *grpE* or both genes encode a negative regulator of the *groE* operon and most probably also of the *dnaK* operon. A more detailed examination of the potential regulatory role of *hrcA* required an *hrcA* null mutation. Therefore, we constructed a *B. subtilis* mutant which carries a large chromosomal deletion within *hrcA* as described in Materials and Methods. The resulting Δ*hrcA* strain was then tested by the slot blot technique for expression of the genes *grpE* and *groEL*, representing the *dnaK* and the *groE* operon, respectively. *B. subtilis* wild type, BT01, and BT02 served as controls (31).

As can be seen from Fig. 1A, lane 4, in the Δ*hrcA* mutant, the *grpE* gene is expressed at a significantly higher level than that of the wild-type strain (lane 1), and there is no further increase in the amount of *grpE*-specific transcript after temperature upshift. The basal level of transcript of the unlinked *groE* operon was also enhanced at low temperature; however, in contrast to *grpE*, there was still a further increase after temperature upshift (Fig. 1B, lane 4).

We conclude from these results that it is *hrcA* rather than *grpE* which codes for a negative regulator of class I heat shock genes. This conclusion was corroborated by the recent finding

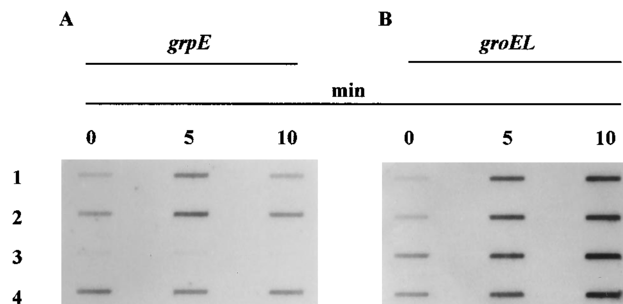

FIG. 1. Transcriptional analysis of different *B. subtilis* strains. Concentration of *grpE* (A) and *groEL* (B) mRNAs. Slot blot analyses of total RNA isolated before (0 min) and after heat shock from 30 to 52°C (5 and 10 min). Lanes: 1, *B. subtilis* 1012 (wild type); 2, *B. subtilis* 1012 BT02 (*dnaK*::*cat*); 3, *B. subtilis* 1012 BT01 (*hrcA*::*cat*); 4, *B. subtilis* 1012 Δ*hrcA*. DIG-labeled oligonucleotides complementary to internal parts of *grpE* (ON2) and *groEL* (ON4) were used as probes. A 1-μg amount of total RNA was applied per slot.

that a *grpE*::*cat* mutant did not influence expression of the class I heat shock genes (16). Furthermore, these results suggest that the *groE* operon is controlled by two different mechanisms, one dependent on and the other independent of *hrcA*.

Measurement of the relative amounts of DnaK and GroEL in two different *B. subtilis* strains. To verify whether the high constitutive level of mRNAs is accompanied by an increased amount of HSPs, the relative amounts of DnaK and GroEL were measured by rocket immunoelectrophoresis (20). Whereas the amounts of DnaK and GroEL increased in the wild-type strain after temperature upshift, there was a significantly higher basal level of both DnaK and GroEL present already at a low temperature in the Δ*hrcA* strain, with no further increase after heat shock in the case of DnaK (Fig. 2A) and a slight increase in the case of GroEL (Fig. 2B). These results clearly confirm those obtained by transcriptional analysis and further strengthen our conclusion that *hrcA* encodes a negative regulator of class I heat shock genes.

After heat shock, the *B. subtilis* Δ*hrcA* strain recovers growth faster than its isogenic wild-type strain. Since in the Δ*hrcA* strain, the *dnaK* and *groE* operons are constitutively expressed at an increased rate already at a low temperature, we asked whether this strain would show altered growth characteristics compared with the wild-type strain and the *hrcA*::*cat* mutant BT01.

We first analyzed growth of the wild-type, BT01, and Δ*hrcA* strains at 30°C (Fig. 3A). Here, all three strains exhibited comparable growth characteristics. In a second experiment, these strains were grown at 30°C till mid-log phase and then shifted to 52°C. Whereas the wild-type strain needed 4 to 5 h to recover from the heat shock, the Δ*hrcA* strain resumed growth already after about 1 h (Fig. 3B). BT01 was not able to resume growth for at least 7 h after temperature upshift. We interpret the growth behavior of the Δ*hrcA* strain to be a consequence of the constitutive high expression of the chaperone genes, thereby resulting in a constitutively increased level of thermotolerance.

Overproduction of HrcA protein prior to heat induction largely prevents increased synthesis of transcripts of the *dnaK* and the *groE* operons. If the HrcA protein acts as a negative regulator of the *dnaK* and *groE* operons, overexpression of this protein prior to heat induction should prevent or at least severely reduce expression of these two operons. To accomplish this goal, the *hrcA* gene was generated by PCR and fused to an IPTG-inducible promoter. The resulting plasmid, p*hrcA*$^+$, was then transformed into the *B. subtilis* wild-type and Δ*hrcA*

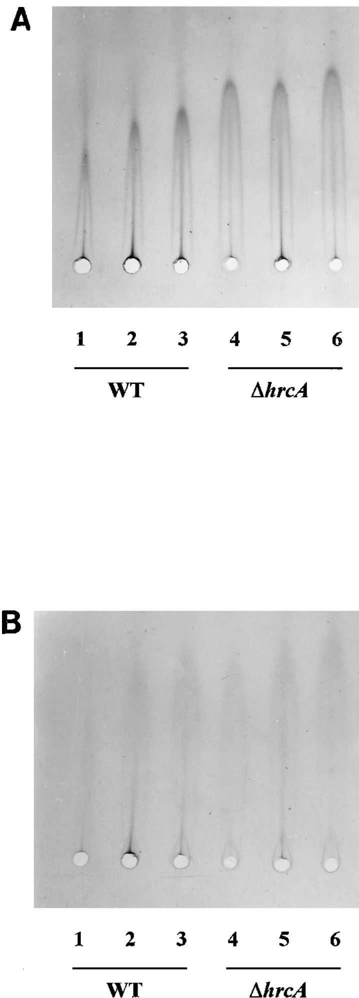

FIG. 2. Relative amounts of DnaK (A) and GroEL (B) in the wild-type (WT) and Δ*hrcA* strains as measured by rocket immunoelectrophoresis. Lanes: 1 to 3, wild-type strain at 0, 10, and 20 min, respectively; 4 to 6, the Δ*hrcA* strain at 0, 10, and 20 min, respectively. Five micrograms of total cell protein was used per lane.

strains, which were subsequently analyzed for transcription of the two operons.

When the *hrcA* oligonucleotide is used as a probe, a dramatic increase in the amount of *hrcA*-specific transcript can be seen after induction of the plasmidal *hrcA*$^+$ gene with IPTG (Fig. 4A, lanes 1 and 4). In the wild-type strain, *hrcA* overexpression allowed only a slight heat induction of the *grpE*, *groES*, and *groEL* transcripts, and the amounts of mRNA were significantly reduced at all times compared with the wild-type situation (Fig. 4B through D; compare lanes 3 and 4). We also analyzed the effect of overproduction of the HrcA protein in the Δ*hrcA* strain (Fig. 4B through D, lanes 1 and 2). Here, the effect was more pronounced than in the wild-type strain. For all genes analyzed, overproduction of HrcA prior to heat shock prevented heat induction completely. These results confirm those obtained with the wild-type strain and add another piece of evidence that *hrcA* encodes a negative regulator of class I heat shock operons.

Overexpression of *hrcA* prevents growth after temperature upshift. We then asked how induction of *hrcA* from p*hrcA*$^+$ prior to heat induction would influence the growth characteristics of wild-type and Δ*hrcA* *B. subtilis* strains. When the two strains, now containing p*hrcA*$^+$, were grown at a low temperature, induction of *hrcA* did not influence growth (Fig. 5A). In contrast, overproduction of *hrcA* followed by heat shock completely prevented growth of the wild-type strain and delayed growth of the Δ*hrcA* strain for at least 5 h (Fig. 5B). These results confirm that HrcA acts as a negative regulator of class I heat shock genes. Its activity is not deleterious to cells at a low temperature, but under heat shock conditions most probably the *dnaK* and *groE* operons and maybe some other not yet identified gene(s) (see Discussion) are not sufficiently expressed.

DISCUSSION

A couple of years ago, we started to analyze the regulation of the heat shock response in *B. subtilis*, the genetic model organism of gram-positive bacteria. One of our motivations was to answer the question of whether the heat shock response is regulated in a way different from what has been reported for *E. coli*. In this bacterium, there are some 31 heat shock genes which are transiently induced after mild heat treatment and which are all under the positive control of the alternate sigma

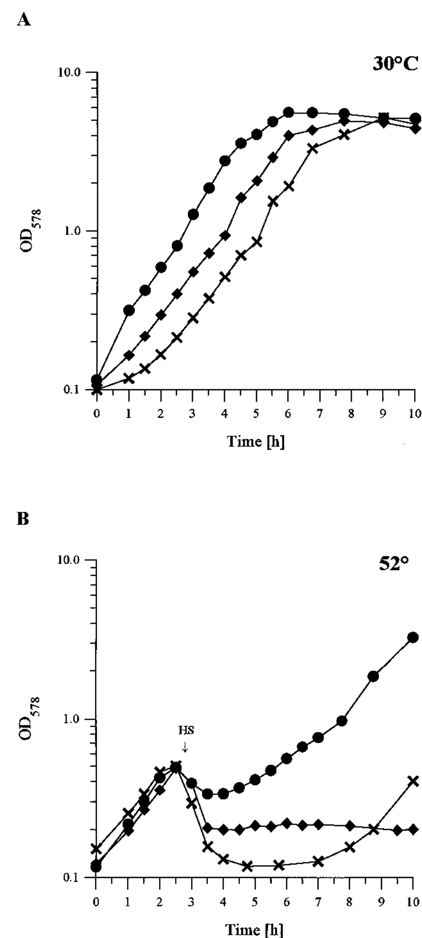

FIG. 3. Growth of three *B. subtilis* strains at two different temperatures. The cells were grown in SMM at 30°C to early log phase, and then the cultures were further incubated at a low temperature (A) or were shifted to 52°C (B). *B. subtilis* wild-type (x), Δ*hrcA* (●), and *hrcA*::*cat* (◆) strains were measured. The arrow indicates the shift of the cultures from 30 to 52°C. OD_{578}, optical density at 578 nm.

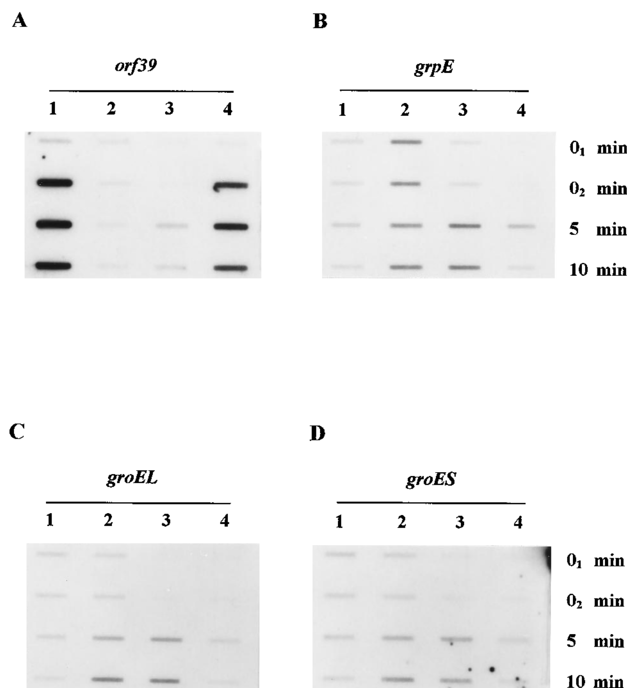

FIG. 4. Transcriptional analysis of different *B. subtilis* strains. The bacterial strains were first grown in LB medium at 30°C till early log phase and were then treated with 2 mM IPTG to induce expression of *hrcA* from the plasmid p*hrcA*$^+$; 30 min later, the cultures were shifted to 52°C. Slot blot analysis of total RNA prepared before the addition of IPTG (0_1), immediately before the temperature upshift (0_2), and 5 and 10 min after heat shock. The DIG-labeled oligonucleotides ON1 (complementary to the 3′ end of *hrcA*, outside the *Hpa*I-*Eco*RV deletion), ON2 (complementary to *grpE*), ON3 (complementary to *groES*), and ON4 (complementary to *groEL*) were used as probes. A 1-μg amount of total RNA was applied per slot. Lanes: 1, *B. subtilis* 1012 Δ*hrcA* containing p*hrcA*$^+$; 2, *B. subtilis* 1012 Δ*hrcA*; 3, *B. subtilis* 1012; 4, *B. subtilis* 1012 containing p*hrcA*$^+$.

factor σ^{32} encoded by the *rpoH* gene (for recent reviews, see references 5 and 39). For approximately 1 year, we know that the situation is completely different in *B. subtilis*. First of all, there are at least three classes of heat shock genes which are transiently induced by mild heat stress (12). We are studying class I heat shock genes, in which we cloned and sequenced the *dnaK* and *groE* operons (29, 36). Both operons are expressed from a vegetative promoter (6), and they are preceded by a perfect inverted repeat of 9 bp separated by a 9-bp spacer which we called CIRCE (41). We could show that introduction of point mutations within the right and left arms and within both arms of the CIRCE element preceding the *dnaK* operon led to a high level of constitutive expression of the *dnaK* operon already at a low temperature (41). We concluded from these results that the CIRCE element acts as a negative *cis* element, most probably as a binding site for a repressor. These data point to a completely novel regulation mechanism for heat shock genes. Moreover, this mechanism seems to be widespread among the eubacteria, since in the meantime the CIRCE element has been described for 29 different bacterial species, always occurring in front of the *dnaK* or *groE* operon. Furthermore, there is now growing evidence that there are bacterial species which contain both the CIRCE element and the *rpoH* gene. Thus far, *Agrobacterium tumefaciens* and *Zymomonas mobilis* have been reported to inherit both the *rpoH* gene (38) and the CIRCE element (2, 32). Whereas the CIRCE element precedes the *groE* operon in both species, the expression of the *dnaK* operons (and most probably that of other heat shock genes) could be directed by σ^{32}.

Subsequently, we attempted to identify the gene(s) encoding the protein(s) interacting with the CIRCE element and to elucidate the complete regulation pathway for the class I heat shock genes. The *dnaK* operon is composed of at least four genes in the transcriptional order *hrcA-grpE-dnaK-dnaJ* (36). Recently, the genomic organization of the *dnaK* operon of *Staphylococcus aureus* has been published elsewhere (23). Here, the structure of the operon is identical to that of *B. subtilis*, but it is followed by an ORF called *orf35*. The deduced amino acid sequence of *orf35* revealed 55% homology with the protein methyltransferase PrmA of *E. coli*, which is responsible for methylation of ribosomal protein L11 (34, 35). In *Clostridium acetobutylicum*, the genomic organization of the *dnaK* operon is identical to that of *B. subtilis*, and here, too, *dnaJ* is followed by an ORF called *orfB* (3). For *B. subtilis*, we have some DNA sequence information for the region downstream of *dnaJ* indicating the beginning of an ORF exhibiting homology with the translation products deduced from *orf35* and *orfB* (19). It is tempting to speculate that these ORFs encode a protein methyltransferase which might be involved in the modification of the repressor. Furthermore, Northern (RNA) blot analysis of the *B. subtilis dnaK* operon revealed an approxi-

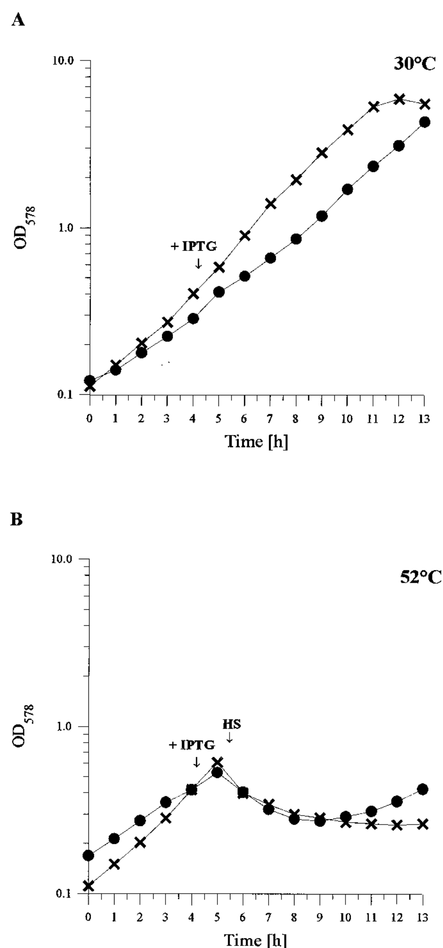

FIG. 5. Growth of two *B. subtilis* strains under different growth regimens. The cells were grown in SMM at 30°C to early log phase, IPTG was added to a final concentration of 2 mM, and then the cultures were further incubated at low temperature (A) or were shifted to 52°C, 30 min after the addition of IPTG (B). x, *B. subtilis* 1012 containing p*hrcA*$^+$; ●, *B. subtilis* Δ*hrcA* containing p*hrcA*$^+$. The arrows indicate induction by IPTG or heat, respectively. OD_{578}, optical density at 578 nm.

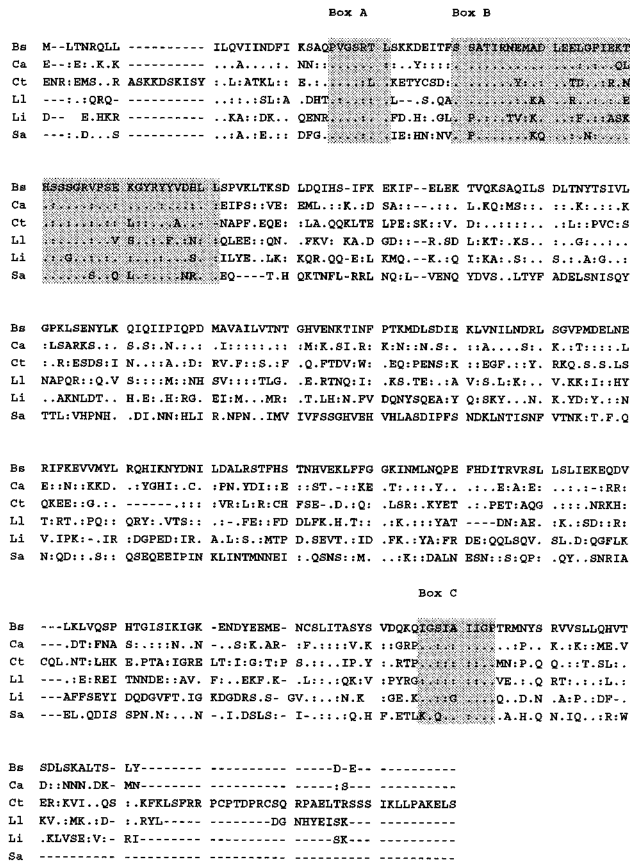

FIG. 6. Alignment of six different *hrcA*-homologous proteins. Bs, *B. subtilis* (36); Ca, *C. acetobutylicum* (3); Ct, *C. trachomatis* (30); Ll, *L. lactis* (8); Li, *Leptospira interrogans* (1); Sa, *S. aureus* (23). Amino acids are listed in the standard one-letter code. Amino acids identical with those of *B. subtilis* are indicated by a point, and conservative replacements (14) are indicated by a colon. Gaps, indicated by dashes, are introduced in order to obtain a maximum fit.

mately 8-kb transcript originating from the vegetative promoter preceding this operon (13). These results suggest that the *dnaK* operon of *B. subtilis* consists of more than five genes, and experiments are in progress to clone and to sequence the downstream region and to mutate these ORFs to elucidate their function.

The experiments reported here clearly demonstrate that the first gene of the *B. subtilis dnaK* operon, *hrcA*, encodes a negative regulator of class I heat shock genes. As mentioned above, in addition to *B. subtilis*, the *dnaK* operons of *S. aureus* and of *C. acetobutylicum* start with an ORF. There are three more examples for an ORF at the beginning of the *dnaK* operon, namely, *Chlamydia trachomatis* (30), *Lactococcus lactis* (8), and *Leptospira interrogans* (1). Do these six genes code for homologous proteins? An alignment of the six proteins deduced from their ORFs exhibits an overall homology of about 30%, but upon a closer look, three regions of extended homology can be deduced, which we call boxes A, B, and C (Fig. 6). It is tempting to speculate that these regions are involved in the activities of these proteins. The high degree of homology of these boxes might allow deduction of primers which can be used to clone *hrcA* homologous genes in cases in which they are not part of the *dnaK* operon, as might be the case for *A. tumefaciens* and *Z. mobilis.*

Does HrcA interact with the CIRCE element? Recently, G. Yuan and S.-L. Wong reported that crude extracts prepared from *E. coli* cells and containing HrcA protein specifically retarded a DNA fragment with the CIRCE element (37). These data strongly suggest that *hrcA* codes for the protein interacting with CIRCE.

Does the Δ*hrcA* strain influence expression only of the *dnaK* and *groE* operons? To answer this question, a two-dimensional protein gel analysis was performed. It turned out that in addition to the protein spots representing the products of the two operons, additional spots show increases in intensity already at a low temperature, suggesting that other, not yet known genes (e.g., those downstream of *dnaJ*) are impaired by the *hrcA* deletion (data not shown). Comparable results have been obtained by analysis of the *hrcA*::*cat* insertion mutant (4).

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