JOURNAL OF BACTERIOLOGY, May 1997, p. 3103–3109 Vol. 179, No. 10
0021-9193/97/$04.00+0

The *htpG* Gene of *Bacillus subtilis* Belongs to Class III Heat Shock Genes and Is under Negative Control

ANJA SCHULZ, SABINE SCHWAB, GEORG HOMUTH, SASKIA VERSTEEG, AND WOLFGANG SCHUMANN*

Institute of Genetics, University of Bayreuth, D-95440 Bayreuth, Germany

Received 2 December 1996/Accepted 5 March 1997

We show that the *htpG* gene of *Bacillus subtilis* is induced by heat, as has been reported for the *Escherichia coli* homolog. Analysis of different mutants revealed that the *htpG* gene belongs to class III heat shock genes in *B. subtilis*. An about 10-fold induction after thermal upshock was found at the levels of both transcription and translation, and this induction resulted from enhanced synthesis of mRNA. By primer extension, we identified one potential transcription start site immediately downstream of a putative σ^A-dependent promoter which became activated after thermal upshift. Northern blot analysis revealed that *htpG* is part of a monocistronic transcriptional unit. An operon fusion where the complete region between *htpG* and its upstream gene was fused to the *bgaB* reporter gene accurately reflected *htpG* expression. Analysis of this fusion revealed that, in contrast to other class III heat shock genes, *htpG* was not induced by osmotic upshock, by ethanol, or by oxygen limitation, suggesting that it belongs to a subgroup within class III. Deletion of the region upstream of the putative promoter resulted in an enhanced basal level of *htpG* expression, but the 10-fold induction was retained, suggesting that the upstream sequences are involved in the regulation of expression in the absence of heat shock.

Organisms as diverse as bacteria, animals, and plants respond to elevated temperatures and to a variety of chemical and physiological stresses by a rapid and transient increase in the synthesis of a set of conserved polypeptides collectively referred to as heat shock proteins (Hsps). The conservation of Hsps between bacteria and eukaryotic organisms suggests that they had an ancient function that was essential for survival throughout evolution. Indeed, most members of the Hsp family are synthesized and accumulate as abundant proteins in the cell even under normal conditions of growth and have essential functions as molecular chaperones involved in protein folding, translocation, higher-order assembly, and protein degradation (4, 8, 9, 12, 25).

Besides elucidating the action of Hsps, another effort focuses on the regulation of the heat shock genes which are tightly controlled at the level of transcription. In *Escherichia coli*, most Hsp genes are under the control of a specific transcription factor, σ^{32}, which directs the bacterial core RNA polymerase to heat shock promoters, and these genes constitute the sigma-32 regulon (for recent reviews, see references 3 and 45). σ^{32} is an unstable protein under normal conditions, and its concentration is transiently increased by changes in translational efficiency and protein stability during heat shock.

In *Bacillus subtilis*, three classes of heat shock genes have been identified and found to be regulated by different mechanisms (for recent reviews, see references 11, 32, and 33). Class I heat shock genes are negatively regulated at the level of transcription, and their regulation involves the HrcA protein (30) interacting with an inverted repeat DNA sequence (6, 44) that we have designated the CIRCE element (46). So far, two operons belonging to class I heat shock genes have been identified: the heptacistronic *dnaK* operon (16) and the bicistronic *groE* operon (21, 37, 41).

Class II heat shock genes are under positive control of the alternate sigma factor σ^B encoded by *sigB*, whose activity is controlled directly by an anti-sigma factor and indirectly by at least six other genes (10, 42). The sigma-B regulon consists of about 40 genes whose induction after thermal upshock is prevented in a *sigB* knockout (11). Heat shock genes belonging to neither class I nor class II, including *clpP* (40), *clpC* (20), *lon* (26), and *ftsH* (5), have been classified as class III. Since regulation of these genes is largely unknown, class III might be heterogeneous (see Discussion).

Recently, the *B. subtilis htpG* gene has been discovered as part of the *Bacillus* genome sequencing project (43). In *E. coli*, *htpG* is part of the sigma-32 regulon and has been identified by a low-stringency hybridization approach using the corresponding *Drosophila* gene as a probe (1). The HtpG protein has about 42% identical amino acid residues with the *Drosophila* and human homologs called Hsp90. Deletion of *htpG* from the *E. coli* chromosome did not effect bacterial growth except at highly elevated temperatures (2). VanBogelen and coworkers showed that *htpG* is induced not only by heat but also by treatment with ethanol, nalidixic acid, or cadmium chloride (39). HtpG was overproduced, purified, and shown to be a dimeric phosphoprotein (35). When cloned on a high-copy-number plasmid, *htpG* can act as a multicopy suppressor of the *secY24* mutation (38). These authors also showed that HtpG accelerated markedly the processing of the outer membrane OmpA porin protein. Very recently, Shirai and coworkers reported that overproduction of HtpG partially suppressed the growth retardation of an *ftsH* mutant (34).

The objective of this study was to analyze the regulation of the *B. subtilis htpG* gene. Here, we show that *htpG* is induced by thermal upshift and that it belongs to class III heat shock genes. Our results further suggest that *htpG* is negatively regulated at the level of transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. To obtain a *sigB*::*cat* derivative of 1012, chromosomal DNA of strain BGH1 (22) was transformed into 1012, and chloramphenicol-resistant transformants were selected. *E. coli* and *B. subtilis* strains were grown aerobically at 37°C in Luria broth (LB) or Spizizen minimal

* Corresponding author. Mailing address: Institute of Genetics, University of Bayreuth, D-95440 Bayreuth, Germany. Phone: 0049 921 552708. Fax: 0049 921 552710.

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
E. coli M15	F^- Sm^r lacZΔM15	14
B. subtilis		
BGH1	*trpC2 lys sigB*::*cat*	22
1012	*leuA8 metB5 trpC2 hsrM1*	27
hrcA::*cat*	1012 with *hrcA*::*cat*	31
Δ*hrcA*	1012 with Δ*hrcA*	30
sigB::*cat*	1012 with *sigB*::*cat*	This work
SS01	1012 with maximum promoter fragment of *htpG* at *amyE*	This work
SS02	1012 with minimal promoter fragment of *htpG* at *amyE*	This work
Plasmids		
pBluescript II SK^+	Ap^r	Stratagene
pBgaB	Ap^r Neo^r promoter test vector	23
pDS56	Ap^r Cm^r His_6 expression vector	14
pDMI.1	Km^r $lacI^q$	14
pDS56-*htpG*	Coding sequence of *htpG* amplified by PCR and cloned into pDS56	This work
p*htpG*	*htpG* amplified by PCR and flanked by *Bam*HI sites, cloned into pBluescript II SK^+	This work
p*htpG*-PEX	370-bp PCR fragment starting upstream of the potential promoter and extending into *htpG*, cloned into pBluescript II SK^+	This work
pAS10	pBgaB with maximum promoter fragment of *htpG*	This work
pAS11	pBgaB with minimal promoter fragment of *htpG*	This work

medium (36). When necessary, LB was supplemented with ampicillin, chloramphenicol, or kanamycin at concentrations of 100, 5, and 10 μg ml^{-1}, respectively.

DNA manipulations and analysis. Plasmid DNA was purified on columns (Qiagen, Hilden, Germany). PCR products were generated by using *Taq* DNA polymerase as specified by the manufacturer (Eurobio, Raunheim, Germany) and using chromosomal DNA of *B. subtilis* as a template. PCR products were purified with a Qiagen PCR purification kit. Cloning was done by standard methods (28). Double-stranded sequencing of plasmids was performed by the dideoxynucleotide chain termination method (29).

Analysis of transcription. Preparation of total RNA, Northern blotting, hybridization, and slot blot analysis were performed as described previously (16). As hybridization probes, we used either the digoxigenin (DIG)-labeled oligonucleotide ON1 (5′-CAAGCGTTTAGACTCTGC-3′), complementary to *htpG* mRNA, or DIG-labeled RNA probes. These probes were synthesized in vitro with T7 RNA polymerase (Boehringer Mannheim DIG-RNA labeling kit) from linearized plasmid p*htpG*, which contains the complete *htpG* gene amplified by the PCR, flanked by *Bam*HI sites and inserted into pBluescript II SK^+. Primer extension was carried out essentially as described previously (44), using the synthetic oligonucleotide ON3 (5′-CAAGCGTTTAGACTCTGC-3′; complementary to the *htpG* transcript) 5′ end labeled with ^{33}P as the primer. Dideoxynucleotide sequencing reactions using the same primer and p*htpG*-PEX were run in parallel to allow determination of the endpoint of the extension product. Plasmid p*htpG*-PEX was obtained by ligating a 370-bp PCR fragment into pBluescript II SK^+. This fragment starts immediately upstream of the σ^A-type promoter and extends into *htpG*.

Overexpression and purification of HtpG and antibody production. To facilitate the overproduction and purification of HtpG, the gene was first amplified by PCR using chromosomal DNA of strain 1012 as a template; both primers had *Bam*HI recognition sequences at their termini. The amplicon was digested with *Bam*HI and cloned into *Bam*HI-linearized pDS56 (14), resulting in pDS56-*htpG*. This plasmid was introduced by electroporation into *E. coli* M15(pDMI.1) for overproduction of His_6-HtpG. This His_6-tagged protein was purified from isopropythiogalactopyranoside (IPTG)-induced cells (2 mM IPTG for 3 h) as described by Hochuli et al. (14) and was used to raise polyclonal antibodies in a rabbit.

Western immunoblot analysis. To visualize the HtpG protein within crude cell lysates of *B. subtilis* cells, we followed a method described previously (15). Filters were incubated with polyclonal primary antibodies at a 1:10,000 dilution. Polyclonal anti-DnaK antibodies were used at a 1:20,000 dilution.

Construction of *htpG-bgaB* operon fusions. Two different *htpG-bgaB* operon fusions were constructed. In one fusion, the potential σ^A-like promoter including a large upstream region (Fig. 1) was ligated to the *bgaB* reporter gene (designated the maximum promoter fragment; the sequence is given in boldface letters in Fig. 1). In the other fusion, the upstream region was omitted, and the promoter region was directly fused to *bgaB* (minimal promoter fragment; corresponds to nucleotides 98 to 153 in Fig. 1). The maximum promoter fragment was generated by PCR using the primers ON2 (5′-GGCCATGGATCCATGAGAA TGAGTGAGAAAAAGAGT-3′) and ON3 (5′-GGCCATGAATTCACCATTA TGTAAACCCTTTTCAGC-3′) and chromosomal DNA as the template. *Bam*HI and *Eco*RI restriction endonuclease sites were incorporated into ON2 and ON3 (underlined), respectively, to facilitate subcloning into the *Bam*HI and *Eco*RI sites of pBgaB (23), creating pAS10. Plasmid pBgaB, a pBR322 derivative, contains the promoterless *bgaB* gene of *Bacillus stearothermophilus* encoding a heat-stable β-galactosidase (13) and a *neo* cassette sandwiched between *amyE*-front and *amyE*-back. The absence of replication in *B. subtilis* allows integration of any DNA sequence at the *amyE* locus of the *B. subtilis* chromosome. The nucleotide sequence of the regulatory region in pAS10 was confirmed by dideoxynucleotide sequencing. The minimal promoter fragment was directly synthesized as two 68-bp oligonucleotides complementary to each other and flanked by *Bam*HI and *Eco*RI sites to facilitate their ligation into *Bam*HI/*Eco*RI-digested pBgaB, resulting in pAS11. The *hptG-bgaB* fusions of pAS10 and pAS11 were then recombined into the *amyE* locus of the strain 1012 chromosome, resulting in strains SS01 and SS02, respectively. Insertion of the fusions at the *amyE* locus in single copy was confirmed by Southern blotting.

β-Galactosidase assays. Cells were grown in LB to mid-log phase at 37°C, BgaB activities were determined as described previously (13), and the activities were expressed as nanomoles of 2-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

RESULTS

The *htpG* gene is induced by heat and belongs to class III heat shock genes. Since *E. coli htpG* has been reported to be a heat shock gene, we first tested whether the *B. subtilis* homolog was also. Total RNA was isolated before and different times

```
     stop orfE3C                        > >>>>> >>
01   TGA TGAAGC CTCTCTTTTT TATGAGAATG AGTGAGAAAA

      >>>>>> >>
40   AGAGTGAAAA CAAATCCAAC TAATGTTAAA TTTACATATA

                        -35
80   TATGCATCTA TCATCTAATT GACAATTGTC ATCTTATGTG

       -10          ↓                        *****
120  ATAAATAGAT GCTGAAAAGG GTTTACATAA TGGTAAAGGA

     *          start htpG
160  GCGATCAAGA GTG
```

FIG. 1. Nucleotide sequence of the region between *orfE3C* and *htpG* (43). The DNA sequence extends from the stop codon of *orfE3C* to the start codon of *htpG*. Indicated are the putative Shine-Dalgarno sequence (asterisks above the sequence), one potential σ^A-dependent promoter, one transcription start site (marked by an arrow), and two direct repeats (arrowheads above the sequence) with significant homology to the right arm of the CIRCE element. The DNA sequence of the maximum promoter fragment is given in boldface letters; that of the minimal promoter fragment corresponds to nucleotides 98 through 153.

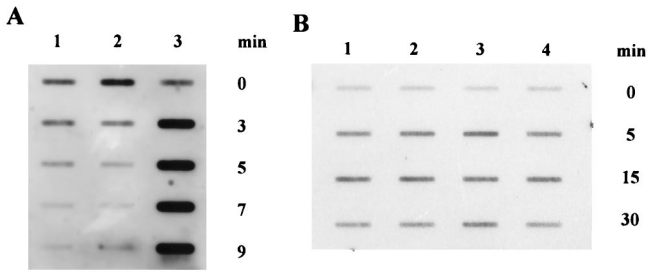

FIG. 2. Slot blot analysis of total RNA isolated before (0 min) and at different times after heat shock from 37 to 48°C as indicated. (A) Rifampin prevents induction of *htpG* mRNA synthesis after thermal upshift. *B. subtilis* 1012 was challenged by rifampin (lane 1), first by rifampin and then by heat (lane 2), and by heat only (lane 3). In lanes 1 and 2, the antibiotic was added at 200 μg ml^{-1} 2 min before thermal upshock. (B) Heat induction of *htpG* occurs independently of *hrcA* or *sigB*. Lanes: 1, *B. subtilis* 1012 Δ*hrcA*; 2, *B. subtilis* 1012 (wild type); 3, *B. subtilis* 1012 *sigB*::*cat*; 4, *B. subtilis* 1012 *hrcA*::cat. One microgram of total RNA was applied per lane. A DIG-labeled riboprobe derived from p*htpG* and complementary to the *htpG* transcript was used as probe.

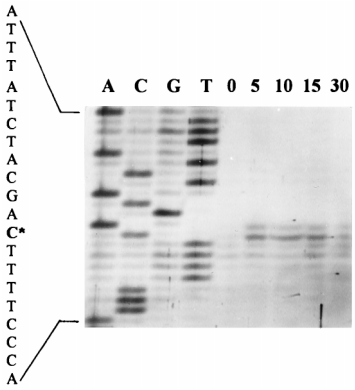

FIG. 3. Mapping of the 5′ end of the *htpG* mRNA by primer extension with ON3. Equal amounts of total RNA (5 μg) isolated from *B. subtilis* 1012 before (0 min) and at the times indicated after temperature upshift were used. The potential start point is marked by an asterisk. Lanes A, C, G, and T show the dideoxy sequencing ladder obtained with ON3 and p*htpG*-PEX as the template.

after exposure to a heat shock from 37 to 48°C, and the amount of *htpG*-specific transcript was measured by slot blotting using a DIG-labeled riboprobe complementary to the *htpG* transcript. As can be seen from Fig. 2B, lane 2, the amount of *htpG* transcript transiently increased after thermal upshift. To determine whether this increase is the result of enhanced synthesis or of increased stability of the transcript, de novo RNA synthesis was blocked 2 min before thermal upshift from 37 to 48°C by the addition of rifampin. The drug not only prevented the increase in *htpG*-specific mRNA after temperature upshift but in addition resulted in a decrease (compare lanes 2 and 3). A similar pattern was observed for a culture not challenged by heat (lane 1). We conclude from these data that *htpG* codes for a heat shock protein and that the increase in the amount of *htpG*-specific transcript results from enhanced synthesis rather than from increased stability of preexisting mRNA as has already been reported for the transcripts of the *dnaK* and *groE* operons (34).

Next, we wished to determine to which class of heat shock genes *htpG* belongs. Class I genes are under negative control by the HrcA protein (30), which binds specifically to an operator sequence called the CIRCE element (6, 44). Inspection of the DNA sequences around the putative σ^A-like promoter failed to detect such an element, suggesting that *htpG* is not a member of class I. To prove this assumption, we analyzed expression of *htpG* in two different *hrcA* knockout mutants, a Δ*hrcA* deletion and an *hrcA*::*cat* insertion mutant. If *htpG* is under *hrcA* control, it should be expressed at a high constitutive rate at low temperatures. The results presented in Fig. 2B, lanes 1 and 4, clearly indicate that expression of *htpG* occurred unimpaired in both null mutants, thereby excluding *htpG* being a member of class I heat shock genes.

Class II genes are under the positive control of the alternate sigma factor σ^B (10). Again, inspection of the DNA sequence upstream of the coding sequence of *htpG* failed to detect a σ^B-like promoter. To assess this observation experimentally, expression of *htpG* was monitored in a *sigB* knockout. As already described for the *hrcA* null mutants, the *htpG* expression pattern before and after heat shock followed that described for the wild-type strain (Fig. 2B, lane 3). These data clearly indicate that transcription of *htpG* is regulated independently of HrcA and of σ^B and therefore *htpG* belongs to class III heat shock genes.

The potential transcription start site of the *htpG* gene suggests a σ^A-type promoter and does not change after heat shock. To map the putative transcription start site(s) of *htpG*, primer extension experiments were performed. Total RNA isolated before and at different times after heat shock was hybridized with ON3, complementary to the noncoding strand at the beginning of *htpG*, and extended with reverse transcriptase. One potential transcription start site was mapped, starting with a G at the level of mRNA, indicating that no additional start site was activated after thermal upshift; the intensity of the signal increased after heat shock (Fig. 3). This putative transcription start site is within the appropriate distance from a potential σ^A-dependent promoter (Fig. 1) and therefore strongly supports our previous suggestion that *htpG* is under the control of only one σ^A-dependent promoter.

The *htpG* gene is part of a monocistronic operon. To determine whether *htpG* is part of a mono- or polycistronic transcriptional unit, total RNA of *B. subtilis* wild-type 1012 was isolated before and after a thermal upshift, separated through a denaturing agarose gel, blotted onto a nylon membrane, and hybridized to a DIG-labeled riboprobe complementary to the *htpG* transcript. The results presented in Fig. 4 revealed the presence of an about 1.9-kb transcript which transiently increased at least 10-fold after heat shock. Since the length of this transcript coincides with that of the *htpG* gene, we conclude that *htpG* is monocistronic at all temperatures.

The amount of HtpG protein raised after heat shock. In another attempt to detect induction of the *htpG* gene after temperature upshift at the level of translation, we determined the amount of HtpG protein by immunoblotting using polyclonal antibodies raised against purified His-tagged HtpG. The results presented in Fig. 5, lane 1, clearly show that the amount of HtpG protein, which was barely visible before thermal upshift, increased significantly after heat treatment. Therefore,

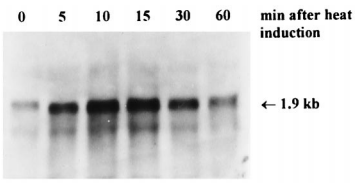

FIG. 4. Northern blot analysis. Total RNAs isolated from *B. subtilis* 1012 at 37°C (0 min) and at the times indicated after exposure to heat stress (48°C; 5, 10, 15, 30, and 60 min) were used. The filter was hybridized with a DIG-labeled riboprobe complementary to *htpG*. Each lane contains 2 μg of RNA.

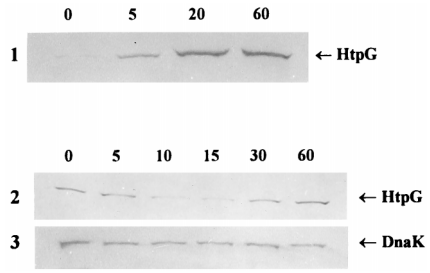

FIG. 5. Immunoblot analyses of heat shock proteins HtpG and DnaK in extracts from *B. subtilis* 1012 treated either with heat or with salt. Lane 1, induction of *htpG* by heat; extracts prepared at 37°C (0 min) and at 5, 20, and 60 min after a temperature shift to 48°C. Treatment of cells with 0.8 M NaCl: lane 2, HtpG; lane 3, DnaK (extracts prepared before [0 min] and at 5, 10, 15, 30, and 60 min after addition of salt).

induction of *htpG* at the level of transcription is followed by increased amounts of protein, confirming that HtpG is a true heat shock protein.

The *htpG* gene is not induced by salt shock. It has been reported that the other members of class III heat shock genes can be induced by stress factors other than heat, e.g., salt shock, oxygen limitation, or ethanol (11). To determine whether *htpG* can be induced by addition of salt, cells were grown in Spizizen minimal medium to mid-logarithmic phase and then challenged with 0.8 M NaCl. Total RNA was isolated before and at different times after addition of salt, and the amount of *htpG*-specific mRNA was determined by slot blotting. In contrast to other members of class III, *htpG* failed to be induced by salt shock (Fig. 6, lane 1). After addition of NaCl, the amount of *htpG*-specific transcript first decreased and then increased again between 15 and 30 min after addition of NaCl and reached a value which might be slightly higher than the preinduction value. Using the same RNA preparation, we also analyzed the induction behavior of class III heat shock genes *ftsH* and *dnaK* as controls. Whereas *ftsH* exhibited a two- to threefold induction in the amount of transcript (Fig. 6, lane 2), in agreement with data already published (5), the amount of *dnaK*-specific transcript remained unchanged after addition of salt, again in agreement with published data that the amount of *dnaK* transcript did not change after an osmotic upshock (40).

These results suggest that *htpG* belongs to those genes whose transcription is reduced for several minutes after the cells have been challenged with salt. Therefore, we were interested to find out whether this drop in transcription was also

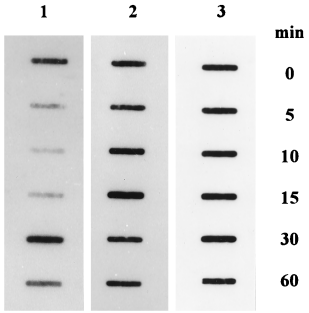

FIG. 6. Transcriptional analyses of different genes after osmotic upshock. Shown are slot blots of total RNA isolated before (0 min) and at different times after addition of 0.8 M NaCl (5, 10, 15, 30, and 60 min). DIG-labeled riboprobes were used as hybridization probes, and 1 μg of RNA was applied per slot. Lane 1, *htpG*; lane 2, *ftsH*; lane 3, *dnaK*.

followed by a drop in the amount of HtpG protein. The amount of HtpG protein in cell lysates was monitored by immunoblotting and revealed a similar drop between 5 and 30 min after addition of NaCl (Fig. 5, lane 2). As a control, we analyzed the amount of DnaK protein in the same lysates, which remained unchanged (Fig. 5, lane 3). These results confirm those obtained by the slot blot analysis and demonstrate that expression of *htpG* is significantly decreased for at least 15 min after application of an osmotic shock.

The *htpG-bgaB* operon fusion accurately reflects *htpG* transcription. To study the transcriptional regulation at the *htpG* locus in *B. subtilis* in more detail, an *htpG-bgaB* operon fusion was constructed by using the maximum promoter fragment. This fusion was recombined in a single copy at the *amyE* locus on the *B. subtilis* chromosome, resulting in strain SS01, which should carry all of the regulatory sequences necessary for expression of *htpG* at a σ^A-type promoter (Fig. 1). To test for the functionality of the *htpG-bgaB* fusion in strain SS01, cells were subjected to a heat shock and β-galactosidase activity was measured before and after thermal upshock. Whereas 5 U of β-galactosidase was found when the cells were grown at 37°C, this activity increased about 10-fold after a shift to 48°C (Fig. 7A). These data are in agreement with those obtained by slot blot, Northern blot, and immunoblot analyses. Thus, the *htpG-bgaB* fusion is an accurate reporter of transcription at the *htpG* locus.

To confirm that *htpG* is not inducible by salt shock, and to analyze the inducibility of the gene by other stresses, we challenged the cells with different stressors known to induce class III heat shock genes (40). Both 0.8 M NaCl and 10% glucose, eliciting an osmotic shock, failed to induce *htpG* (Fig. 7A); the same result was obtained with 5% ethanol and oxygen limitation. These data suggest that *htpG* is member of a subgroup of class III heat shock genes inducible by heat but not by other stresses that are known to induce the other class III heat shock genes.

***htpG* is under dual control.** Experiments with strain SS01 carrying the maximum promoter region have shown that this DNA fragment most probably contains all sequences necessary for heat shock regulation. To identify those sequences that might be involved in this regulation, a shorter promoter fragment was fused to *bgaB* (minimal promoter fragment) and recombined at the *amyE* locus (strain SS02). This fusion starts with the potential σ^A-type promoter, thereby being devoid of all the upstream sequences. Analysis of this operon fusion in response to heat shock resulted in a significant difference compared to the maximum promoter fragment (Fig. 7B). The basal level of β-galactosidase was increased 3- to 4-fold, resulting in about 15 U, followed by an about 10-fold induction after thermal upshock. We conclude from these results that the upstream region contains DNA sequences responsible for low-level expression at normal temperatures. DNA sequences important for heat induction either overlap with the putative σ^A-type promoter or are situated downstream of the promoter. Challenging strain SS02 with other stress factors did not result in any induction of *htpG*, as already reported for the fusion with the maximum promoter fragment (Fig. 6B).

DISCUSSION

Transcriptional regulation of the *htpG* gene of *B. subtilis* was studied. Results of slot blot and Northern blot analyses and of primer extension clearly showed that the amount of *htpG* mRNA transiently increased about 10-fold after thermal upshock. This could result from stabilization of the preexisting transcripts, from enhanced transcription, or from a mixture of

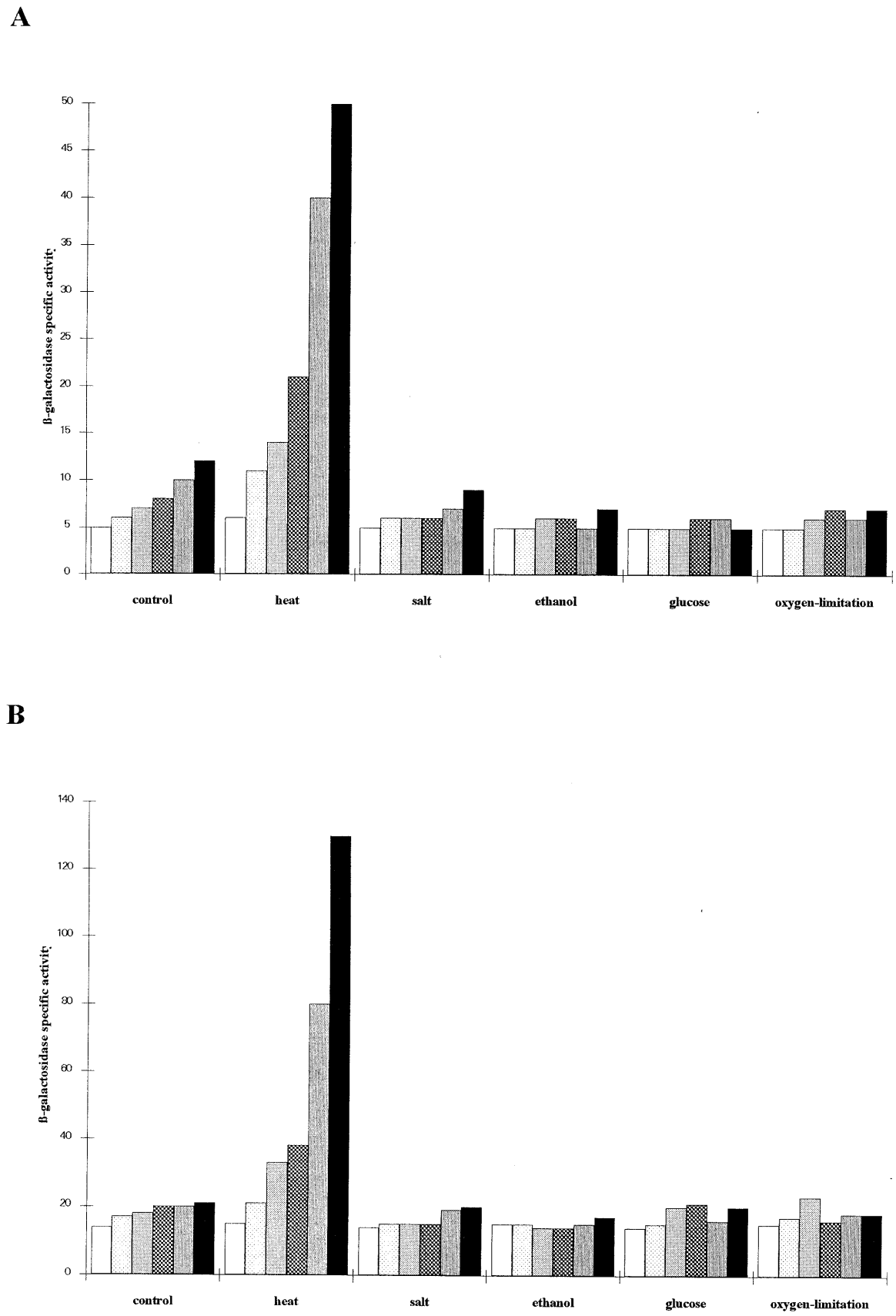

FIG. 7. Heat but none of several other stress factors induces the *htpG* gene. (A) Strain SS01 carrying the maximum promoter fragment (A) and strain SS02 containing the minimum promoter fragment (B) were incubated in LB at 37°C and challenged with different stress factors. Values (left to right) represent β-galactosidase activities before exposure (open bars) and after 5 (dotted bars), 10 (light grey bars), 15 (cross-hatched bars), 30 (light grey bars), or 60 (solid bars) min of exposure to the stress indicated below the bars. Control, incubation at 37°C; heat, exposure to 48°C; salt, 0.8 M NaCl; ethanol, 5%; glucose, 10%; oxygen limitation (40).

both. To distinguish between these possibilities, de novo synthesis of RNA was blocked by the addition of rifampin shortly before the cells were challenged with heat. This treatment completely abolished any increase in the amount of *htpG*-specific transcript after thermal upshift. Therefore, this increase results from enhanced transcription rather than stabilization of preexisting *htpG* mRNA.

Analysis of transcription in null mutants of *hrcA* and *sigB* identified *htpG* as a novel member of class III heat shock genes. This finding is strongly supported by the result of primer extension analysis which identified a single potential transcription start site located in an appropriate distance downstream of a putative σ^A-dependent promoter. The existence of a third class of heat shock genes has been deduced from two different observations. First, a deletion of *sigB* did not affect the induction of a few proteins by heat, as visualized on two-dimensional protein gels (40). Second, some of these genes have been cloned and sequenced, and neither the CIRCE element nor a potential σ^B-type promoter was found. Instead, they are preceded by a σ^A-type promoter recognized by the vegetative sigma factor. So far, all code for ATP-dependent proteases or ATPases, namely, ClpP (40), ClpC (20), Lon (26), and FtsH (5).

At the moment, the definition of class III heat shock genes is as follows: (i) they are controlled neither by the HrcA repressor nor by the alternative sigma factor σ^B; (ii) they are expressed from a σ^A-dependent promoter; and (iii) they are induced by a variety of stress factors such as ethanol, puromycin, salt, or oxidative stress. Most probably, a more detailed analysis of the regulation of the members of class III will lead to a redefinition of this class. The first example is *clpC*, part of an operon containing six genes (24) where σ^A- and σ^B-dependent promoters were mapped upstream of the first gene (19). Whereas a strong induction by heat, ethanol, and salt stress occurred at the σ^B-dependent promoter, the vegetative promoter was induced by hydrogen peroxide or puromycin. In a *sigB* mutant, the σ^A-dependent promoter became inducible by heat and ethanol stress, thereby compensating for the *sigB* deficiency (19). The second example is *htpG*, since this gene was not induced by ethanol, osmotic stress, or oxygen limitation as shown here. In summary, the results published by Krüger and coworkers (19) and those presented here demonstrate that all members of class III are not regulated by the same mechanism as has been reported for those of class I and class II.

Expression of *htpG* seems to be under dual control. This assumption is based on the analyses of two promoter fragments of different lengths fused to the *bgaB* reporter gene. In both cases, exposure of cells carrying the transcriptional fusions to heat resulted in an about 10-fold induction of β-galactosidase activity, in agreement with data obtained by direct measurement of *htpG* mRNA and HtpG protein. Induction of *htpG* might occur either by inactivation of a repressor as reported for class I heat shock genes or by activation of a transcriptional activator. A second layer of regulation seems to influence the basal level of *htpG* expression and needs DNA sequences upstream of the putative σ^A-dependent promoter. This conclusion is drawn from the observation that the basal level of β-galactosidase measured with an operon fusion devoid of the promoter upstream sequences is increased by a factor of about 3. Here, too, the protein(s) interacting with these upstream sequences is unknown. It should be mentioned that this upstream region contains a direct repeat with significant homology to the right arm of the CIRCE element (seven of nine nucleotides identical in both repeats). Whether this direct repeat plays any role in the regulation of the basal level of transcription is an open question at the moment. At least our analysis of *htpG* transcription in the two *hrcA* null mutants did not provide any hint that *hrcA* might be involved. The elucidation of this new mechanism of heat stress induction is currently under investigation.

What is the biological function of the HtpG protein within the cell before and after heat stress? As already mentioned, an *E. coli htpG* null mutant had no phenotype, and *htpG* affected bacterial growth only at elevated temperatures (2). Experiments to isolate an *htpG* null mutant in *B. subtilis* are in progress. The only thing that we know already is that *htpG* is not an essential gene, because there is one *B. subtilis* strain with a large chromosomal deletion called Δ*igf* which includes *htpG*, and this strain is viable (7). We tested whether overproduction of HtpG will influence growth of *B. subtilis* cells at 37°C. The *htpG* gene was fused to an inducible promoter as part of a high-copy-number plasmid, and addition of the inducer to cells did not alter bacterial growth (18). In addition, overproduction of HtpG protein did not alleviate growth retardation of an *ftsH* null mutant as has been reported for *E. coli* (34). In eukaryotes, the role of Hsp90 is not completely clear, although it is the most abundant cytosolic Hsp and has an essential function in *Saccharomyces cerevisiae*. Hsp90 binds unfolded polypeptides and either silences their function (e.g., steroid receptor protein), helps them to fold properly, or escorts them to their proper cellular compartment (e.g., $pp60^{v\text{-}src}$) (for a recent review, see reference 17).

ACKNOWLEDGMENTS

Financial support was obtained from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. **Bardwell, J. C. A., and E. A. Craig.** 1984. Major heat shock gene of *Drosophila* and the *E. coli* heat inducible *dnaK* gene are homologous. Proc. Natl. Acad. Sci. USA **81:**848–852.
2. **Bardwell, J. C. A., and E. A. Craig.** 1988. Ancient heat shock gene is dispensable. J. Bacteriol. **170:**2977–2983.
3. **Bukau, B.** 1993. Regulation of the heat-shock response. Mol. Microbiol. **9:**671–680.
4. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: Molecular chaperones of protein biogenesis. Microbiol. Rev. **57:**402–414.
5. **Deuerling, E., B. Paeslack, and W. Schumann.** 1995. The *ftsH* gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshock. J. Bacteriol. **177:**4105–4112.
6. **Emmerich, R.** Unpublished results.
7. **Fujita, Y., and T. Fujita.** 1983. Genetic analysis of a pleiotropic deletion mutation (Δ*igf*) in *Bacillus subtilis*. J. Bacteriol. **154:**864–869.
8. **Georgopoulos, C., and W. J. Welch.** 1993. Role of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol. **9:**601–634.
9. **Gething, M.-J., and J. Sambrook.** 1992. Protein folding in the cell. Nature **355:**33–45.
10. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59:**1–30.
11. **Hecker, M., W. Schumann, and U. Völker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417–428.
12. **Hendrick, J. P., and F.-U. Hartl.** 1993. Molecular chaperone functions of heat-shock proteins. Annu. Rev. Biochem. **62:**349–384.
13. **Hirata, H., S. Negoro, and H. Okada.** 1985. High production of thermostable β-galactosidase of *Bacillus stearothermophilus* in *Bacillus subtilis*. Appl. Environ. Microbiol. **49:**1547–1549.
14. **Hochuli, E., W. Bannwarth, H. Döbeli, R. Gentz, and D. Stüber.** 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. Bio/Technology **6:**1321–1325.
15. **Homuth, G., M. Heinemann, U. Zuber, and W. Schumann.** 1996. The genes *lepA* and *hemN* form a bicistronic operon in *Bacillus subtilis*. Microbiology **142:**1641–1649.
16. **Homuth, G., S. Masuda, A. Mogk, Y. Kobayashi, and W. Schumann.** 1997. The *dnaK* operon of *Bacillus subtilis* is heptacistronic. J. Bacteriol. **179:**1153–1164.
17. **Jakob, U., and J. Buchner.** 1994. Assisting spontaneity: The role of Hsp90 and small Hsps as molecular chaperones. Trends Biochem. Sci. **19:**205–211.
18. **Jekle, A.** Unpublished results.
19. **Krüger, E., T. Msadek, and M. Hecker.** 1996. Alternate promoters direct

stress-induced transcription of the *Bacillus subtilis clpC* operon. Mol. Microbiol. **20:**713–724.
20. **Krüger, E., U. Völker, and M. Hecker.** 1994. Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. J. Bacteriol. **176:** 3360–3367.
21. **Li, M., and S.-L. Wong.** 1992. Cloning and characterization of the *groESL* operon from *Bacillus subtilis*. J. Bacteriol. **174:**3981–3992.
22. **Maul, B., U. Völker, S. Riethdorf, S. Engelmann, and M. Hecker.** 1995. σ^B-dependent regulation of *gsiB* in response to multiple stimuli in *Bacillus subtilis*. Mol. Gen. Genet. **248:**114–120.
23. **Mogk, A., R. Hayward, and W. Schumann.** 1996. Integrative vectors for constructing single-copy transcriptional fusions between *Bacillus subtilis* promoters and various reporter genes encoding heat-stable enzymes. Gene **182:**33–36.
24. **Ogasawara, N., S. Nakai, and H. Yoshikawa.** 1994. Systematic sequencing of the 180 kilobase region of the *Bacillus subtilis* chromosome containing the replication origin. DNA Res. **1:**1–14.
25. **Parsell, D. A., and S. Lindquist.** 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. **27:**437–496.
26. **Riethdorf, S., U. Völker, U. Gerth, A. Winkler, S. Engelmann, and M. Hecker.** 1994. Cloning, nucleotide sequence, and expression of the *Bacillus subtilis lon* gene. J. Bacteriol. **176:**6518–6527.
27. **Saito, H., T. Shibata, and T. Ando.** 1979. Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **170:**117–122.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **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.
31. **Schulz, A., B. Tzschaschel, and W. Schumann.** 1995. Isolation and analysis of mutants of the *dnaK* operon of *Bacillus subtilis*. Mol. Microbiol. **15:**421–429.
32. **Schumann, W.** 1996. The heat shock stimulon of *Bacillus subtilis*. Braz. J. Genet. **19:**387–398.
33. **Schumann, W.** 1996. Regulation of the heat shock response in *Escherichia coli* and *Bacillus subtilis*. J. Biosci. **21:**133–148.
34. **Shirai, Y., Y. Akiyama, and K. Ito.** 1996. Suppression of *ftsH* mutant phenotypes by overproduction of molecular chaperones. J. Bacteriol. **178:**1141–1145.
35. **Spence, J., and C. Georgopoulos.** 1989. Purification and properties of the *Escherichia coli* heat shock protein, HtpG. J. Biol. Chem. **264:**4398–4403.
36. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44:**407–408.
37. **Tozawa, Y., H. Yoshikawa, F. Kawamura, M. Itaya, and H. Takahashi.** 1992. Isolation and characterization of the *groES* and *groEL* gene of *Bacillus subtilis* Marburg. Biosci. Biotechnol. Biochem. **56:**1995–2002.
38. **Ueguchi, C., and K. Ito.** 1992. Multicopy suppression: an approach to understanding intracellular functioning of the protein export system. J. Bacteriol. **174:**1454–1461.
39. **VanBogelen, R. A., P. M. Kelley, and F. C. Neidhardt.** 1987. Differential induction of heat shock, SOS, and oxidative stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. **169:**26–32.
40. **Völker, U., S. Engelmann, B. Maul, S. Riethdorf, A. Völker, R. Schmid, H. Mach, and M. Hecker.** 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology **140:**741–752.
41. **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.
42. **Yang, X., C. M. Kang, M. S. Brody, and C. W. Price.** 1996. Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. Genes Dev. **10:**2265–2275.
43. **Yoshida, K., S. Seki, M. Fujimura, Y. Miwa, and Y. Fujita.** 1995. Cloning and sequencing of a 36-kb region of the *Bacillus subtilis* genome between the *gnt* and *iol* operons. DNA Res. **2:**61–69.
44. **Yuan, G., and S. Wong.** 1995. Isolation and characterization of *Bacillus subtilis* 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.
45. **Yura, T., H. Nagai, and H. Mori.** 1993. Regulation of the heat-shock response in bacteria. Annu. Rev. Microbiol. **47:**321–350.
46. **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.