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

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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 upshift 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^32^-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 *hgaB* 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 upshift, 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 upshift 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 affect 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...
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 Tag 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 probe. These probes were synthesized in vitro using T7 RNA polymerase (Boehringer Mannheim DIG-RNA labeling kit) from linearized plasmid pbgGB, which contains the complete htpG gene amplified by the PCR, flanked by BamHI sites and inserted into pBluescript II SK⁺. Primer extension was carried out essentially as described previously (44), using the synthetic oligonucleotide ON1 (5'-CAAGCGTTTAGACTCTGC-3'; complementary to the htpG transcript) 5' end labeled with 32P as the primer. Dideoxynucleotide sequencing reactions using the same primer and pbgGB-PEX were run in parallel to allow determination of the endpoint of the extension product. Plasmid pbgGB-PEX was obtained by ligating a 370-bp PCR fragment into pBluescript II SK⁺. This fragment starts immediately upstream of the α-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 BamHI recognition sequences at their termini. The amplicon was digested with BamHI and cloned into BamHI-linearized pDS56 (14), resulting in pDS56-htpG. This plasmid was introduced by electroporation into E. coli htpG homolog by gap-recombination (27).

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. BgaB activities were determined as described previously (13), and the activities reported correspond to the start codon of htpG. Indicated are the putative Shine-Dalgarno sequence (asterisks above the sequence). The DNA sequence extends from the stop codon of orfE3C to the start codon of htpG, which is marked by an arrow. 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.

### 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 of temperature stress.
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 operon sequence called the CIRCE element (6, 44). Inspection of the DNA sequences around the putative σ^H^-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 ΔhrcA 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^A^ (10). Again, inspection of the DNA sequence upstream of the coding sequence of *htpG* failed to detect a σ^A^-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 a σ^H^- 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 *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 *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,

![Figure 2](http://jb.asm.org/)

![Figure 3](http://jb.asm.org/)

![Figure 4](http://jb.asm.org/)
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 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 recombinated 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 σ70-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 recombinated at the amyE locus (strain SS02). This fusion starts with the potential σ70-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 σ70-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 $\sigma^B$-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 $\sigma^B$-type promoter was found. Instead, they are preceded by a $\sigma^B$-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 $\sigma^A$; (ii) they are expressed from a $\sigma^B$-dependent promoter; and (iii) they are induced by a variety of stress factors such as ethanol, purumycin, 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 $\sigma^A$ and $\sigma^B$-dependent promoters were mapped upstream of the first gene (19). Whereas a strong induction by heat, ethanol, and salt stress occurred at the $\sigma^B$-dependent promoter, the vegetative promoter was induced by hydrogen peroxide or puromycin. In a sigB mutant, the $\sigma^B$-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 $\beta$-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 $\sigma^B$-dependent promoter. This conclusion is drawn from the observation that the basal level of $\beta$-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 a 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 $\sigma^A$g 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 anftsH 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$^{src}$) (for a recent review, see reference 17).

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