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Heat Shock Proteins Do Not Influence Wet Heat Resistance of *Bacillus subtilis* Spores

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Spores of *Bacillus subtilis* are significantly more resistant to wet heat than are their vegetative cell counterparts. Analysis of the effects of mutations in and the expression of fusions of a coding gene for a thermostable β-galactosidase to a number of heat shock genes has shown that heat shock proteins play no significant role in the wet heat resistance of *B. subtilis* spores.

The gram-positive bacterium *Bacillus subtilis* undergoes the process of sporulation when nutrients become exhausted, and the resulting spores are more resistant than are the growing cells to a variety of environmental insults including heat, UV and gamma radiation, and a number of toxic chemicals (8, 34). Wet heat resistance is probably the most dramatic resistance property of dormant spores, as spores are resistant to about 40°C-higher temperatures than are vegetative cells (8). Spore wet heat resistance is due to a number of factors including dehydration of the spore protoplast or core (8), mineralization of the spore core (8), saturation of spore DNA with α/β-type small acid-soluble proteins (33, 34), and thermal adaptation, as spores of a single species formed at higher temperatures are more wet heat resistant than are spores formed at lower temperatures (8, 39).

Although many factors contribute to spore wet heat resistance, the identity of the target for wet heat killing of spores is not known. However, two different types of studies have indicated that spore DNA is not the killing target and suggested that some spore protein might be the target (2, 7, 30). If spore killing by wet heat is indeed through protein damage, then it is possible that repair or removal of a damaged protein might be important in spore wet heat resistance. Proteins that can repair or remove denatured proteins in vivo are often members of the heat shock regulon, which is important in the survival of many different bacteria after a heat shock (9, 17). Since sporulation at elevated temperatures results in spores with increased heat resistance and heat shock protein synthesis is increased at elevated temperatures (12, 38), then spores prepared at higher temperatures may also have increased levels of heat shock proteins which may in turn contribute to their increased heat resistance. In order to investigate whether proteins of the heat shock regulon play any role in wet heat resistance of *B. subtilis* spores, we have examined (i) the effect of mutations in known heat shock genes on spore wet heat resistance, (ii) the effect of mild heat shock at various times during sporulation on spore wet heat resistance, and (iii) the expression of heat shock genes during germination of spore populations which had been killed ~50% by wet heat treatment.

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Effects of mutations in heat shock genes on spore wet heat resistance. The heat shock genes of *B. subtilis* are grouped into at least three classes based on the precise mechanism for the regulation of their expression (11); we examined the effects of mutations in representatives from each of the three classes. Mutations in class I genes included a polar mutation in *dnaK*, a polar mutation in *hrcA*, and a nonpolar mutation in *hrcA*. The mutated class II gene was *sigB* (14) encoding the RNA polymerase sigma factor, σ^B, which directs transcription of other class II genes; consequently, a mutation in *sigB* abolishes transcription of all class II genes (1, 3, 4, 38). Mutations in class III genes included a mutation in *lonA* (24, 26) and a nonpolar mutation in *ctsR* (6); *ctsR* is a negative regulator of the *clpP*, *clpC*, and *clpE* operons (5, 6, 15), so a mutation in *ctsR* results in the overexpression of those operons. We had hoped to also study strains with a mutation in *clpC*, but such strains sporulated extremely poorly, as noted previously (22).

All of the mutations noted above were introduced into our wild-type *B. subtilis* (PS832) background and into the isogenic strain (termed $\alpha^-\beta^-$) lacking the genes, *sspA* and *sspB*, that encode the two major α/β-type small acid-soluble proteins (PS356) (19) (Tables 1 and 2). The *ctsR* mutant strain was constructed by congression of plasmid pHTΔctsR along with the *cat* marker in chromosomal DNA of strain QB4903, since pHTΔctsR does not carry an antibiotic resistance marker (6). Because a number of the mutations that we wished to analyze were available, we had only to construct the polar mutation in *dnaK* and the polar and nonpolar mutations in *hrcA*. For construction of the *dnaK* mutation, a DNA fragment containing the 5′ end of the *dnaK* gene (−186 to +74 relative to the *dnaK* translation start site [+1]) was PCR amplified from strain PS832 chromosomal DNA with primers ΔdnaK1w and ΔdnaK1x, and the PCR product was cut with *Hin*dIII (site within ΔdnaK1w) and *Eco*RI (site within ΔdnaK1x) and cloned between the same sites in plasmid pJL74 (16) to generate plasmid pdnaK1. The 3′ end of the *dnaK* gene (+1740 to +2037 relative to the *dnaK* translation start site [+1]) was amplified similarly with primers ΔdnaK2y and ΔdnaK2z, and the PCR product was cut with *Bam*HI (site within ΔdnaK2y) and *Eag*I (site within ΔdnaK2z) and cloned between the same sites in plasmid pdnaK1 to generate plasmid pdnaK1/2. In this plasmid, the two cloned PCR products flank a spectinomycin resistance (Sp^r) marker. For construction of the polar *hrcA* mutation, a DNA fragment containing the 5′ end of the *hrcA*

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TABLE 1. Plasmids used

Plasmid	Description (antibiotic resistance)	Source or reference
pPctc-bgaB	Derivative of pMLK83 carrying a *ctc-bgaB* fusion (Km^r)	W. Schumann
pPclpC-bgaB	Derivative of pMLK83 carrying a *clpC-bgaB* fusion (Km^r)	W. Schumann
pPlon-bgaB	Derivative of pMLK83 carrying a *lonA-bgaB* fusion (Km^r)	W. Schumann
pPdnaK-bgaB	Derivative of pMLK83 carrying a *dnaK-bgaB* fusion (Km^r)	20
pDL2/pgroE-bgaB	Derivative of pDL carrying a *groEL-bgaB* fusion (Cm^r)	40
pHTΔctsR	Derivative of pHT315 used in introducing Δ*ctsR* (Cm^r)	6
pJL74	Shuttle plasmid replicating in both *E. coli* and *B. subtilis* (Ap^r Sp^r)	16
pdnaK1/2	Derivative of pJL74 used to generate Δ*dnaK* strains (Sp^r)	This work
phrcA1/2	Derivative of pJL74 used to generate the polar Δ*hrcA* mutation (Sp^r)	This work
phrcA-np	Derivative of pJL74 used to generate the nonpolar Δ*hrcA* mutation (Sp^r)	This work

gene (−208 to +236 relative to the *hrcA* translational start site [+1]) was PCR amplified from strain PS832 chromosomal DNA with primers ΔhrcA1w and ΔhrcA1x, and the PCR product was cut with *Hin*dIII (site within ΔhrcA1w) and *Eco*RI (site within ΔhrcA1x) and cloned between the same sites in plasmid pJL74 (16) to generate plasmid phrcA1. The 3′ end of the *hrcA* gene (+988 to +1184 relative to the *hrcA* translational start site [+1]) was amplified in the same manner with primers ΔhrcA2y and ΔhrcA2z, cut with *Bam*HI (site within ΔhrcA2y) and *Eag*I (site within ΔhrcA2z), and cloned between the same sites in plasmid phrcA1 to generate phrcA1/2, in which the two cloned PCR products flank a Sp^r marker. In order to construct a nonpolar mutation in *hrcA*, this gene's promoter and translation start site plus an in-frame stop codon were placed immediately before the downstream *grpE* gene which is cotranscribed with *hrcA*, resulting in production of a truncated HrcA protein (16 amino acids as opposed to 343 amino acids in the wild-type protein) while still allowing translational coupling of *hrcA* to the remainder of the operon. A DNA fragment containing 500 bp from *hemN*, the gene upstream of *hrcA* (+592 to +1092 relative to the *hemN* translational start site [+1]) was PCR amplified from strain PS832 chromosomal DNA with primers Δhem/A and Δhem/B, and the PCR product was cut with *Hin*dIII (site within Δhem/A) and *Eco*RI (site within Δhem/B) and cloned between the same sites in plasmid pJL74 (16) to generate plasmid pJLhem. A DNA fragment containing the promoter region and a small fragment of the 5′ end of *hrcA* (−190 to +33 relative to the *hrcA* translational start site [+1]) was amplified similarly with primers ΔphrcAlong and ΔphrcBlong, and the PCR fragment was cut with *Bam*HI (site within ΔphrcAlong) and *Eag*I (site within ΔphrcBlong) and cloned between the same sites in plasmid pJLhem to generate plasmid pJLhemhrc. Finally, a DNA fragment containing the translational start site of the *grpE* gene immediately downstream of *hrcA* (−76 to +506 relative to the *grpE* translational start site [+1]) was amplified with primers Δpgrp/A and Δpgrp/B, and the PCR fragment was cut with *Eag*I (site within Δpgrp/A) and *Sst*I (site within Δpgrp/B) and cloned between the same sites in plasmid pJLhemhrc to generate plasmid phrcA-np, in which the *hemN* and *hrcA* fragments flank a Sp^r marker and the *grpE* fragment is cloned downstream of the truncated *hrcA* gene. The sequences of the primers used in

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TABLE 2. *B. subtilis* strains used

Strain	Genotype and phenotype	Source or reference[a]
ML6	*sigB*::*cat* Cm^r	14
PS356	Δ*sspA* Δ*sspB* $\alpha^-\beta^-$	19
PS832	Wild-type, *trp*$^+$ revertant of strain 168	Laboratory stock
PS2538	*sigB*::*cat* Cm^r	ML6→PS832
PS2539	*sigB*::*cat* Δ*sspA* Δ*sspB* $\alpha^-\beta^-$ Cm^r	ML6→PS356
PS2542	Δ*lon*::*cat* Cm^r	RS359→PS832
PS2543	Δ*lon*::*cat* Δ*sspA* Δ*sspB* $\alpha^-\beta^-$ Cm^r	RS359→PS356
PS2567	*amyE*::*groEL-bgaB* Cm^r	WBG2→PS832
PS2643	*amyE*::*ctc-bgaB* Km^r	pPctc-bgaB→PS832
PS2645	*amyE*::*dnaK-bgaB* Km^r	pPdnaK-bgaB→PS832
PS2647	*amyE*::*clpC-bgaB* Km^r	pPclpC-bgaB→PS832
PS2549	*amyE*::*lon-bgaB* Km^r	pPlon-bgaB→PS832
PS3044	Δ*dnaK*::*spc* Sp^r	pdnaK1/2→PS832
PS3045	Δ*dnaK*::*spc* Δ*sspA* Δ*sspB* $\alpha^-\beta^-$ Sp^r	pdnaK1/2→PS356
PS3046	Δ*hrcA*::*spc* Sp^r	phrcA1/2→PS832
PS3047	Δ*hrcA*::*spc* Δ*sspA* Δ*sspB* $\alpha^-\beta^-$ Sp^r	phrcA1/2→PS356
PS3085	*amyE*::*clpP′-bgaB cat* Δ*ctsR* Cm^r	QB4903 + pHTΔctsR→PS832
PS3086	*amyE*::*clpP′-bgaB cat* Δ*ctsR* Δ*sspA* Δ*sspB* $\alpha^-\beta^-$ Cm^r	QB4903 + pHTΔctsR→PS356
PS3302	Δ*hrcA*::*spc* Sp^r	phrcA-np→PS832
PS3303	Δ*hrcA*::*spc* Δ*sspA* Δ*sspB* $\alpha^-\beta^-$ Sp^r	phrcA-np→PS356
QB4903	*amyE*::*clpP′-bgaB cat trpC2* Cm^r	6
RS359	Δ*lon*::*cat* Cm^r	26
WBG2	*amyE*::*groEL-bgaB* Cm^r	40

[a] DNA(s) from the plasmid or strain left of the arrow was used to transform the bacterial strain to the right of the arrow.

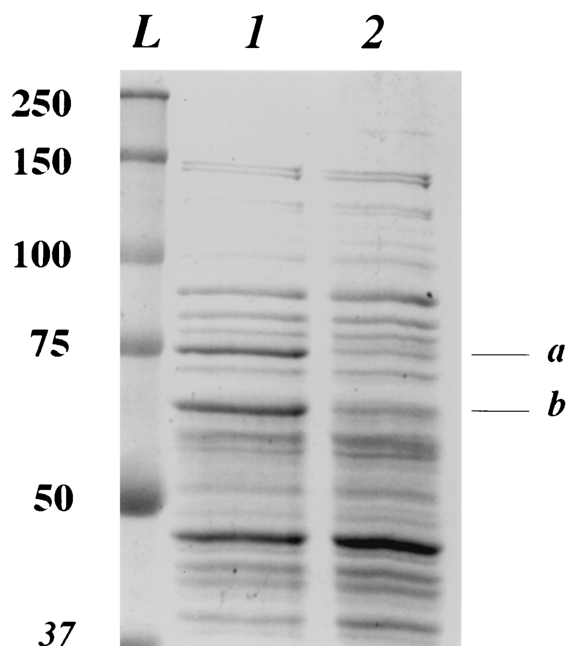

FIG. 1. SDS-polyacrylamide gel electrophoresis analysis of extracts from wild-type (PS832) and nonpolar *hrcA* mutant (PS3032) *B. subtilis* spores. Spores were decoated, washed, and lysed as described in the text; 25 μg of protein was run on an SDS–10% polyacrylamide gel; and the gel was stained with Coomassie blue. Lane L contains molecular mass markers whose kilodaltons are given on the left side of the gel, lane 1 is the *hrcA* spore extract, and lane 2 is wild-type spore extract. The bands labeled a and b are DnaK and GroEL, respectively.

these PCRs are available upon request. After confirmation of the expected DNA sequence in these plasmids, they were used to transform *B. subtilis* strains PS832 and PS356 to Sp^r (100 μg/ml). Southern blot analysis of appropriately digested chromosomal DNA from Sp^r transformants confirmed that the clones used for further analysis had the indicated deletions (Tables 1 and 2).

Since the *hrcA* gene is a negative regulator of class I gene expression including that of *dnaK* and the *groESL* operons, a nonpolar mutation in *hrcA* should result in the overexpression of all of these genes, while a polar mutation in *hrcA* should result in the overexpression of just the *groESL* operon, since *hrcA* is the first gene in the *dnaK* operon (27, 40). In order to demonstrate this point directly, cleaned spores (optical density at 600 nm [OD_{600}] of ~30) (see below) were decoated with 1 ml of 0.1 M NaOH–0.1 M NaCl–0.5% sodium dodecyl sulfate (SDS)–0.1 M dithiothreitol for 2 h at 37°C; washed 10 times by centrifugation with 1 ml of H_2O; and suspended in 500 μl of 25 mM Tris-HCl (pH 7.5)–5 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride–40 μg of lysozyme. After incubation for 5 min at 37°C and 30 min at 4°C, the extract was centrifuged, an aliquot of the supernatant fluid was run on an SDS-polyacrylamide gel, either the gel was stained with Coomassie blue or the proteins were transferred to nitrocellulose-based paper, and DnaK and GroEL were detected using anti-*Escherichia coli* GroEL (Sigma) or anti-*Chlamydia trachomatis* DnaK (a gift of Svend Birkelund) antisera (10). These analyses showed that the *hrcA* polar and nonpolar mutations did indeed result in overexpression of GroEL or DnaK and GroEL, respectively, in both vegetative cells and spores (Fig. 1 and data not shown). Presumably, the nonpolar *hrcA* mutation also caused overexpression of GroES, but we have not yet shown this directly.

Spores of all strains were prepared at 37°C in 2× SG medium (23) without antibiotics, cleaned as described previously (23), and stored in water at 10°C; all spores whose resistance was to be compared were prepared, cleaned, and tested together. All spore preparations used were free (>98%) of vegetative or sporulating cells, germinated spores, and cell debris. In order to determine the spore titer, an aliquot (100 μl) of spores at an OD_{600} of 1 was diluted in distilled water and multiple samples of several dilutions were plated on Luria-Bertani medium plates (18, 25) containing kanamycin (10 μg/ml), spectinomycin (100 μg/ml), or chloramphenicol (5 μg/ml) as needed. The remaining spores at an OD_{600} of 1 were incubated at 90°C (wild-type strains) or 85°C ($\alpha^-\beta^-$ strains) for various times, and aliquots were removed, diluted, and plated as described above. Plates were incubated at 37°C for 18 h prior to enumeration. All experiments were performed on at least two independent spore preparations, and all spore preparations were tested at least twice.

With one exception (see below), the wet heat resistance of spores from strains with mutations in heat shock genes was essentially identical to that of the parental spores (Table 3). Although there was slight variability in spore heat resistance between different experiments and spore preparations, when spores were tested and prepared together, the relative heat resistance of wild-type and mutant spores was essentially identical. The only mutant spores which differed significantly in heat resistance from that of wild-type spores were *sigB* spores, which had a small but reproducibly lower wet heat resistance compared with that wild-type spores. This effect of the *sigB* mutation was even more dramatic in the $\alpha^-\beta^-$ genetic background (Table 3). Density gradient centrifugation of decoated spores as described previously (37) showed that there were no

TABLE 3. Wet heat resistance of spores of various strains[a]

Expt no.[b]	Heat shock gene mutated	Wild-type background, D_{90}-min[c] (strain)	$\alpha^-\beta^-$ background, D_{85}-min[c] (strain)
1	None	14 (PS832)	9 (PS356)
1	*dnaK*	14 (PS3044)	8 (PS3045)
2	None	14 (PS832)	12 (PS356)
2	*hrcA* (polar)	14 (PS3046)	10 (PS3047)
3	None	13 (PS832)	13 (PS356)
3	*hrcA* (nonpolar)	12 (PS3302)	15 (PS3303)
4	None	8 (PS832)	6 (PS356)
4	*lon*	7 (PS2542)	6 (PS2543)
5	None	7 (PS832)	14 (PS356)
5	*sigB*	5 (PS2538)	4 (PS2539)
6	None	12 (PS832)	15 (PS356)
6	*ctsR*	11 (PS3085)	13 (PS3086)

[a] Spores were prepared and cleaned and wet heat resistance was measured as described in the text.
[b] Spores used in individual experiments were from separate preparations, but within an experiment, spores from different strains were prepared and tested together.
[c] Time in minutes to kill 90% of spores at either 90 or 85°C.

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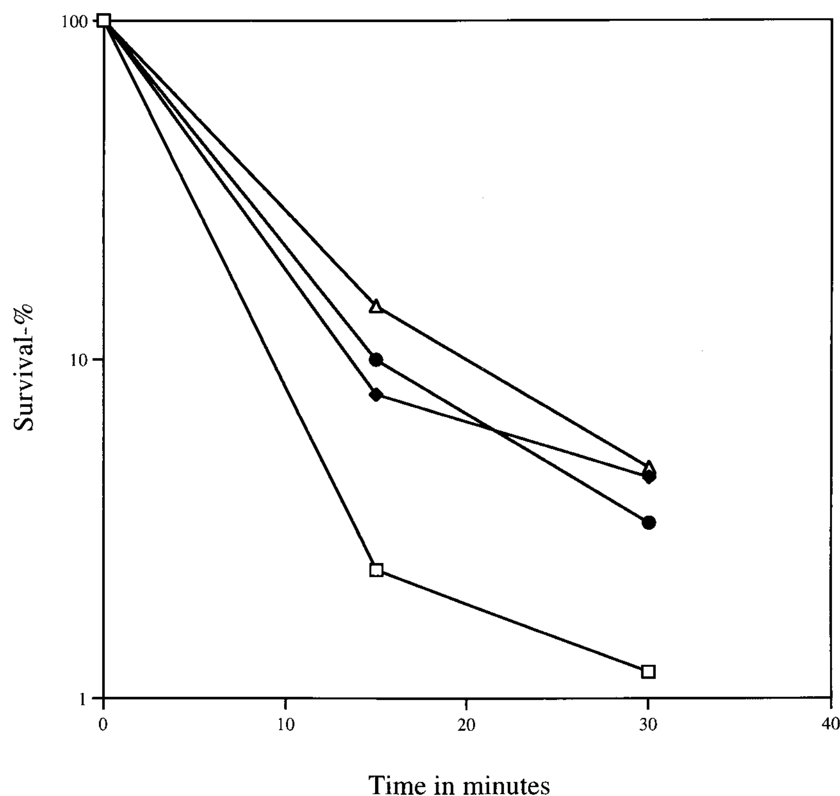

FIG. 2. Heat resistance of spores from wild-type *B. subtilis* cultures shifted from 30 to 45°C for 30 min during sporulation. Sporulation of strain PS832 was in resuspension medium with the time of initiation of sporulation defined as the time of resuspension. Spores were cleaned and wet heat resistance at 90°C was measured as described in the text. Similar results were obtained in experiments performed twice, as well as during sporulation in nutrient exhaustion (2× SG) medium. The symbols used are as follows: □, unshifted culture; ◆, culture shifted at the initiation of sporulation; △, culture shifted at the second hour of sporulation; ●, culture shifted at the fourth hour of sporulation. Error bars have been omitted for clarity, but the spores produced in the culture that was shifted at the second hour of sporulation had D_{90} values (see Table 3) that were from 28 to 110% (±10%) greater in multiple experiments than values for spores from unshifted cultures or from cultures shifted earlier or later in sporulation.

differences in core water content between the *sigB* and parental spores with either wild-type or $\alpha^-\beta^-$ backgrounds (data not shown). In addition, we found that both wild-type and $\alpha^-\beta^-$ spores exhibited the same mutation frequency, ~4.5% auxotrophic or asporogenous colonies among survivors of wet heat treatments giving 90% killing (7). We also measured the dry heat resistance of *dnaK*, *sigB*, *ctsR*, and PS832 spores at 120°C as described previously (32) and again found no differences (data not shown). It is important to note again that the nonpolar *hrcA* mutant overexpresses DnaK and GroEL in spores (Fig. 1), and presumably also GroES. However, these spores had the same wet heat resistance as did the wild-type spores (Table 3), indicating that overexpression of class I heat shock proteins does not affect spore heat resistance.

Effect of heat shock during sporulation on spore wet heat resistance. As noted above, it is known that, when cultures of the same strain are sporulated at different temperatures, the spores from cultures sporulated at the higher temperature are more wet heat resistant than are those sporulated at lower temperatures (8, 39). It has also been reported previously that when *Bacillus megaterium* or *B. subtilis* cultures at 27 or 30°C were shifted to 45 or 48°C for 30 min at 1 to 2 h into sporulation, the resultant spores were more heat resistant than were those from cultures which had not been subjected to a temperature shift or had been shifted earlier or later in sporulation (21, 28), and we obtained similar results. Cultures were sporulated at 30°C by the resuspension method (36) to ensure the maximum synchrony of the sporulation process, shifted to 45°C for 30 min at various times during sporulation, and returned to 30°C for the remainder of sporulation, and the heat resistance of the resulting spores was measured (Fig. 2). The same experiment was also performed during sporulation in 2× SG medium (23) (data not shown). In both cases, spores from cultures that were shifted to 45°C at various times in sporulation were more heat resistant than were those from cultures not shifted at all, with cultures shifted 2 h into sporulation consistently giving spores with slightly more heat resistance (Fig. 2 and data not shown). We also used Western blot analysis to examine the level of DnaK and GroEL in spores from cultures sporulated at 30°C in 2× SG medium and either shifted to 45°C for 30 min or not shifted. Cleaned spores (OD_{600} of ~75) were lyophilized and dry ruptured for 8 min with glass beads as the abrasive, and the dry powder was suspended in 500 μl of cold 25 mM Tris-HCl (pH 7.4)–5 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride. After incubation for 30 min at 4°C, the extract was centrifuged, an aliquot of the supernatant fluid was run on an SDS-polyacrylamide gel, proteins were transferred to nitrocellulose-based paper, and

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DnaK and GroEL were detected as described above. There was no (<15%) difference in the levels of either GroEL or DnaK in the more heat-resistant spores from the appropriately heat-shocked culture compared to the spores from the non-heat-shocked culture (data not shown). These results agree with those of a recent study using two-dimensional gel electrophoresis (21) which found a transient, but no permanent, increase in the levels of a number of heat shock proteins in cells from sporulating cultures that had been heat shocked. These latter results thus strongly suggest that increased spore levels of heat shock proteins are not responsible for the increased heat resistance of spores from appropriately heat-shocked cultures. To prove this point conclusively, we performed the same temperature shift during sporulation of *sigB* and *dnaK* strains. Again, spores from cultures of these mutant strains that were shifted to 45°C at ~2 h into sporulation exhibited increased wet heat resistance compared to that of spores from unshifted cultures (data not shown), strongly indicating that heat shock proteins are not involved in the elevated wet heat resistance of spores from cultures subjected to a heat shock. We also analyzed the core wet density (37) of spores from cultures which had or had not been subjected to a 30-min shift from 30 to 45°C at the second hour of sporulation. Since these values were both 1.355 ± 0.01 g/ml, there was no difference in the core wet density and thus the core water content of these spores, indicating that this is not the cause of the increased heat resistance of spores from appropriately heat-shocked sporulating cultures (8).

Expression of heat shock genes during germination of heat-treated spores. The results given above strongly suggest that heat shock proteins play no significant role in spore wet heat resistance and suggest that repair or removal of heat-damaged proteins during spore germination and outgrowth is not important to spore survival after heat treatment. Heat shock of growing cells is known to induce the synthesis of a number of heat shock proteins in a variety of species (17). If removal or renaturation of damaged proteins is not important in spore heat resistance, then wet heat treatment of dormant spores would not result in the production of heat shock proteins during subsequent spore germination. In order to test this prediction, we examined the expression of a number of heat shock genes during germination of wet heat-treated spores. The genes examined were *groEL*, *dnaK*, *ctc*, *lonA*, and *clpC*, and their expression was monitored by measuring β-galactosidase synthesis from transcriptional fusions in which the promoter of the gene in question was fused to the promotorless *bgaB* gene encoding the thermostable β-galactosidase from *Bacillus stearothermophilus*; these *bgaB* fusions were inserted into the *amyE* locus on the *B. subtilis* chromosome (20). If any of these gene products are important in repairing or removing a heat-damaged protein, we would expect to see an increase in β-galactosidase synthesis from the *bgaB* fusion during germination of heat-treated spores compared to synthesis in germinating unheated spores (31). Cleaned spores carrying the various *bgaB* fusions were prepared at 37°C in 2× SG medium (23) and treated with wet heat to give ~50% killing. Both heated and unheated spores (OD_{600} of 20) were germinated at 37°C in 25 ml of Spizizen's minimal medium (35) plus 0.1% Casamino Acids and also containing 5 μCi of [^{3}H]leucine in order to measure total protein synthesis (31). Samples were taken throughout germination and outgrowth (~3 h), β-galactosidase activity and total protein synthesis were determined, and the β-galactosidase specific activity was calculated relative to total protein synthesized (31), since there is essentially no β-galactosidase from any of these *bgaB* fusions in spores (data not shown). The β-galactosidase specific activity would be higher in the culture from heated spores if expression of the heat shock gene in question had been induced during germination and outgrowth by prior spore heat treatment (31). However, upon analysis of the expression of all five heat shock genes, we found less than a 25% difference in the β-galactosidase specific activities in germinating-outgrowing cultures from heated versus unheated spores (data not shown). In contrast, in vegetatively growing cells, heat shock results in a 4- to 25-fold induction of expression of these same genes (11, 24, 38). From these data, we conclude that the heat shock genes that we tested are not induced by prior heat treatment of spores.

Conclusions. The findings in this work allow three major conclusions. First, as reported by two other groups (13, 21, 28), a heat shock at an early time in sporulation results in an increase in wet heat resistance of the resultant spores. This effect does not appear to involve the heat shock response, as there was no elevation in the level of heat shock proteins in the spores with elevated wet heat resistance, as also found in a recent study (21), and we also found that mutations in several heat shock genes did not abolish this phenomenon. While the specific reason for the effect of a heat shock at the second hour of sporulation is not clear, it may be simply the result of a minor, albeit global, alteration in transcription at a key time in sporulation which results in production of spores with slightly altered properties, including slightly increased wet heat resistance. Indeed, recent work has indicated that global alterations in transcription during sporulation can significantly alter spore properties (29).

The second conclusion is that a *sigB* mutation has a significant effect on spore heat resistance, with this effect being greater in an $\alpha^-\beta^-$ background. Our studies also show that this effect is not to due to increased transcription of heat shock genes by σ^B. The specific reason for this effect is not clear, although it may well result from a subtle alteration in transcription of multiple genes during sporulation.

The third conclusion from this work is that heat shock proteins appear to play no role in spore wet heat resistance. A role for the heat shock regulon in spore wet heat resistance has been suggested previously (28), but our findings clearly show that (i) mutations in heat shock genes (with the exception of *sigB*) do not alter spore wet heat resistance, (ii) loss-of-function mutations in heat shock genes do not eliminate the increase in wet heat resistance of spores from heat-shocked cultures, (iii) expression of heat shock genes is not induced during germination of wet heat-treated spores, and (iv) overexpression of class I heat shock genes does not result in increased spore heat resistance. We have clearly shown that class I and class II heat shock genes are not involved in spore heat resistance, since a mutation in *dnaK* and overexpression of class I genes do not affect spore heat resistance and neither *groEL* nor *dnaK* is transcribed during germination of heat-treated spores. In addition, mutation of *sigB*, the gene encoding the σ factor necessary for the transcription of all class II

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genes, results in only a slight reduction in spore heat resistance, which is likely due to an indirect effect on sporulation. We have not tested mutations in all possible heat shock genes, as mutations in some of these genes are lethal or abolish sporulation. Thus, it is not possible to definitively rule out all class III heat shock genes as playing a role in spore heat resistance. However, our analyses of the major players in the heat shock response in vegetative cells strongly indicate that the heat shock response as it functions in growing cells plays no role in spore wet heat resistance.

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