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Expression of a Stress- and Starvation-Induced *dps/pexB*-Homologous Gene Is Controlled by the Alternative Sigma Factor σ^B in *Bacillus subtilis*

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σ^B-dependent general stress proteins (Gsps) of *Bacillus subtilis* are essential for the development of glucose-starvation-induced cross-resistance to oxidative challenge. However, the proteins directly involved in this nonspecific resistance to oxidative stress have to be identified. We found that one prominent Gsp displayed strong sequence similarity to the previously characterized oxidative-stress-inducible MrgA protein of *B. subtilis* and to the starvation-induced Dps/PexB protein of *Escherichia coli*. We therefore designated this prominent Gsp Dps. While MrgA belongs to the peroxide-stress-inducible proteins needed for the H_2O_2-inducible adaptive response to oxidative stress, Dps belongs to the proteins induced by heat, salt, or ethanol stress and after starvation for glucose but not by a sublethal oxidative challenge. Primer extension experiments identified two overlapping promoters upstream of the coding region of *dps*, one being σ^B dependent (P_B) and the other being σ^B independent (P_1). Both promoters contribute to the basal level of *dps* during growth. After stress or during entry into the stationary phase, transcription from P_B strongly increased whereas transcription from P_1 decreased. Mutant strains lacking Dps completely failed to develop glucose-starvation-induced resistance to oxidative stress. These results confirm our suggestion that σ^B-dependent general stress proteins of *B. subtilis* are absolutely required for the development of nonspecific resistance to oxidative stress.

As has been described for *Escherichia coli* cells (20), *Bacillus subtilis* cells respond to the deleterious effects of hydrogen peroxide in two different ways. Exposure of exponentially growing cells to sublethal levels of hydrogen peroxide confers protection against subsequent treatment with lethal doses of hydrogen peroxide (32). This inducible resistance to oxidative stress is caused by the specific induction of genes of the peroxide regulon, including the genes encoding vegetative catalase (KatA), alkyl hydroperoxide reductase (AhpCF), and a DNA-binding and -protecting protein (MrgA) (14, 15, 21).

In contrast, stationary-phase cells which are starved for glucose are constitutively resistant to lethal H_2O_2 levels (16). The alternative sigma factor σ^B was found to be strongly activated after stress or during glucose starvation (7, 10). More than 60 general stress proteins which are strongly induced by heat, salt, and ethanol stress or on entry into the stationary phase provoked by starvation for glucose belong to this large σ^B-dependent-stress and stationary-phase regulon (8, 11, 22, 40). Despite the large number of σ^B-dependent proteins identified so far (11, 22, 40), *sigB* mutants showed no detectable growth or sporulation phenotype (9, 17, 24, 26). Because of their nonspecific induction profile, σ^B-dependent proteins, like the "post-exponential starvation" proteins of *E. coli*, are thought to provide nongrowing cells with a general resistant state (22, 31). This assumption gained support by the finding that the σ^B-dependent catalase KatE was highly homologous to the σ^s-dependent catalase HPII of *E. coli*, indicating that σ^B-dependent proteins might be associated with the general resistant state to oxidative stress at the onset of starvation (19, 28). Consistent with this idea, *sigB* mutants failed to develop starvation-induced cross-resistance to oxidative stress (18). Surprisingly, the σ^B-dependent catalase KatE does seem not to be required for this starvation-mediated oxidative-stress resistance (18). Therefore, we attempted to identify further σ^B-dependent stress proteins which might be responsible for this nonspecific resistance to oxidative stress.

N-terminal sequencing of selected protein spots from two-dimensional (2-D) gels allowed the identification of a general stress protein of *B. subtilis* which displayed a high degree of similarity to the σ^s-dependent Dps protein from *E. coli* (1, 29). In this paper we describe the expression of *dps* of *B. subtilis* in response to different types of stress and starvation for glucose. Furthermore, we found that strains lacking the *dps* gene failed to develop starvation-induced resistance to lethal doses of hydrogen peroxide.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used were *B. subtilis* 168 (*trpC2*) (4), ML6 (*trpC2 sigB*::Δ*Hin*dIII-*Eco*RV::*cat*) (24), BGH9 (*trpC2 dps*::Δ*Acc*I-*Hin*dIII::*neo*), and *E. coli* DH5α and RR1. *E. coli* strains were routinely grown in a complex medium and used as hosts for DNA manipulation. *B. subtilis* strains were cultivated under vigorous agitation at 37°C in a synthetic medium containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4 \cdot 7H_2O$, 27 mM KCl, 7 mM sodium citrate × $2H_2O$, 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2 \times 2H_2O$, 1 μM $FeSO_4 \times 7H_2O$, 10 μM $MnSO_4 \times 4H_2O$, 4.5 mM glutamic acid, 780 μM tryptophan, 860 μM lysine, and 0.2% (wt/vol) glucose (38). For heat shock, cells were shifted from 37 to 48°C. The other stress conditions were achieved by exposing cells to either 4% (wt/vol) NaCl, 4% (vol/vol) ethanol, or 60 μM H_2O_2. Starvation for glucose was provoked by cultivating the bacteria in a medium containing limiting amounts of glucose (0.05%, wt/vol).

Survival assays after H_2O_2 challenge. In order to determine the oxidative-stress resistance at different stages of growth, cells were cultivated in a synthetic medium under conditions of glucose starvation. At an optical density of 0.4 at 500 nm, 5 ml of the culture was placed in new Erlenmeyer flasks and challenged with 10 mM H_2O_2. Appropriate dilutions of the H_2O_2-treated and untreated cultures were plated on agar plates 10 min after the addition of H_2O_2. Aliquots

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FIG. 1. Alignment of *B. subtilis* Dps (Dps Bac.su.) with homologous DNA binding proteins of the Dps family: DpsA of *Synechococcus sp.* PCC7942 (Dps Syn.sp.) (accession no. A44631 [34]); the fine tangled major pilus subunit of *Haemophilus ducrei* (pil.su.Hae.ducr) (accession no. U18769); the hypothetical protein of *Haemophilus influenza* (YD49 Hae.infl) (accession no. p45173); the neutrophil-activating protein (bacterioferritin) of *Helicobacter pylori* (NapA Hel.pyl.) (accession no. p43313); MrgA of *B. subtilis* (MrgA Bac.su.) (accession no. p37960 [13]); the hypothetical protein in the bromoperoxidase 5′ region of *Streptomyces aureofaciens* (YBP2 Str.au.) (accession no. p29714 [35]); the cold shock protein of *Anabena variabilis* (YLT2 Ana.va.) (accession no. p29712); Dps/PexB protein of *E. coli* (Dps E.coli) (accession no. p27430 [1]); and the antigens from *Treponema pallidum* (TYF1 and TPF2 Tre.pa.) (accession no. p17915 and p16665 [33]). Black shadows indicate identical amino acids, and grey shadows indicate similar amino acids.

of the growing culture were treated with H_2O_2 at the time points indicated in Fig. 5 until bacteria reached the stationary phase. The number of CFU of the untreated culture was considered 100%.

Analytical and preparative 2-D PAGE and N-terminal sequencing. Analytical 2-D polyacrylamide gel electrophoresis (PAGE) was performed with bacterial cell extracts which were labeled with L-[^{35}S]methionine for 3 min before and 10 min after exposure to ethanol and oxidative stress according to the method of Bernhardt et al. (8) by using the 2-D Investigator System of Oxford Glycosystem. For N-terminal sequencing, protein samples were separated by preparative 2-D PAGE and the protein spots that were collected from several 2-D gels were concentrated, blotted, and sequenced as described previously (5).

Construction of a *dps* deletion mutant. For construction of a *dps* deletion mutant, the gene was amplified from chromosomal DNA of *B. subtilis* 168 with the oligonucleotide primers Mdps1, 5′ AGAGTGATTTTCTTGTGG 3′, and Mdps2, 5′ TATCCATTTATCCCAACA 3′. The PCR product was ligated with the *Xho*I- and *Eco*RI-digested vector pBluescriptII KS(+), which resulted in plasmid pKSd1. This plasmid was digested with *Acc*I and *Hin*dIII and ligated with the *neo* cassette from pBEST501 (25) digested with *Sma*I, which generated plasmid pKSdn11. The *Sca*I-linearized plasmid pKSdn11 was used to transform competent cells of *B. subtilis* 168. Neomycin-resistant colonies were selected on agar plates containing 10 μg of neomycin per ml. The mutation was verified by PCR with the primers Mdps1 and Mdps2.

Analysis of transcription. Total RNA of the *B. subtilis* strains was isolated from exponentially growing cells before and after their exposure to stress by the acid phenol method of Majumdar et al. (30) with modifications as described previously (40).

Northern blot analyses were performed as described previously (41). Hybridization specific for *dps* was conducted with a digoxigenin-labeled RNA probe synthesized in vitro with T7 RNA polymerase (noncoding strand) from *Hin*dIII-linearized plasmid pKSDps1. This plasmid contains the 200-bp internal fragment of *dps* which was amplified from chromosomal DNA with the forward primer DpsHind1 (5′ CCCAAGCTTGGGTGGTATGTGAAAGGGAAAG ATT 3′) and the reverse primer DpsBam (5′ CGGGATCCCGTTGAACCATT TGTTCAGCTG 3′). The PCR product was cut with *Hin*dIII and *Bam*HI and ligated with pBluescriptII KS(−) digested with the same enzymes.

For primer extension experiments, the synthetic oligonucleotide primer complementary to the N-terminus-encoding region of the *dps* gene (DPS4, 5′ ATC TTTCCCTTTCACATACC 3′) was labeled with [γ-^{32}P]ATP at its 5′ end and used as the primer for the primer extension analysis as described previously (41). Sequencing of the *dps* promoter region was performed with plasmid pKSdps2, containing the 543-bp PCR product amplified with the primers DpsHind2 (5′ CCCAAGCTTGGGAGAGTGATTTTCTTGTGGTGG 3′) and DpsBam, as the template. Sequencing was performed with the primer DPS4 according to the method of Sanger et al. (37).

General methods. Plasmid isolation, restriction enzyme analysis, transformation of *E. coli*, ligation of DNA fragments, and filling in of the recessed 3′ termini by the Klenow fragment of DNA polymerase I were performed according to standard protocols (36). Transformation of natural competent *B. subtilis* cells was carried out as described by Hoch (23).

Computer analysis of sequence data. Database searches were performed with the BLAST program (2).

Nucleotide sequence accession numbers. The nucleotide sequence data reported here have been deposited in the EMBL and GenBank nucleotide sequence databases under accession no. AF008220. The N-terminal protein sequence of Gsp20u appears in the SwissProt database under the accession no. p80879.

RESULTS

Identification of Dps on 2-D gels by N-terminal sequencing. One of the most prominently labeled σ^B-dependent general stress proteins synthesized after stress or starvation displayed a molecular mass of nearly 18 kDa and was designated Gsp20u (6). N-terminal sequencing from a Coomassie blue-stained 2-D gel allowed the determination of the sequence for Gsp20u (SEQLIQAVNKQVAN) (6), which matches the sequence of the hypothetical protein YtkB of *B. subtilis* (predicted molecular mass, 16.59 kDa; predicted isoelectric point, 4.64). Database searches of YtkB showed significant similarity to the MrgA protein of *B. subtilis* (12, 13) and to the *E. coli* Dps/PexB protein (1), indicating that Gsp20u belongs to the family of Dps-homologous proteins, which are present in a variety of organisms (33–35) (Fig. 1). The Dps-encoding gene *ytkB* was sequenced in the course of the *B. subtilis* genome sequencing project (accession no. AF008220) and maps at 268° on the genetic map of *B. subtilis* (27). DNA sequence analysis revealed the presence of a rho-independent terminator immediately downstream of *dps*. Upstream of the *dps* gene, there is an open reading frame (*ytkA*) (Fig. 2) whose product displays similarity to a metalloproteinase precursor protein (data not shown).

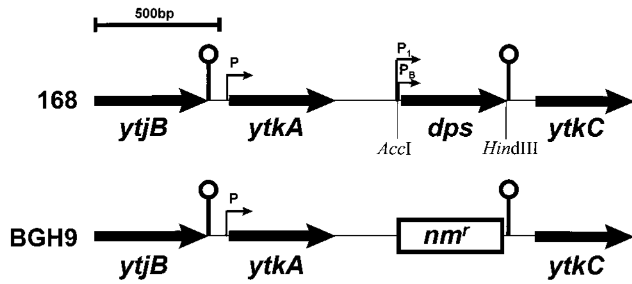

FIG. 2. Physical organization of the *dps* (*ytkB*) region. For construction of the dps deletion mutant BGH9, the *dps* gene has been replaced by an Nm^r cassette as described in Materials and Methods.

Dps and MrgA are differentially regulated. According to our 2-D gel analysis, the spot corresponding to Dps was induced after heat, salt, and ethanol stress or glucose starvation in a σ^B-dependent manner but was not induced by sublethal levels of H_2O_2 (Fig. 3). In contrast, MrgA was strongly induced in response to oxidative stress but not in response to other types of stress and therefore belongs to the peroxide-specific-stress proteins (12). Whereas the MrgA spot was detected in its monomeric as well as its oligomeric complex form on the 2-D gels, we were not able to detect a multimeric form of Dps on the 2-D gels.

Transcription of *dps* is induced by stress or starvation for glucose in a σ^B-dependent manner. As shown in Fig. 4A, we detected one main transcript of 500 bp which was strongly increased in the wild type after heat, salt, and ethanol stress as well as after glucose starvation. On Northern blots with RNA of a *sigB* deletion mutant, a weak 500-bp signal which was weakly increased after heat shock but was not induced after salt and ethanol stress or glucose starvation was detected. This finding indicates that transcription of *dps* occurs in a monocistronic manner from two promoters; one is σ^B dependent, and the other is σ^B independent. In addition, there was also a weak signal at 1.2 kb which was not induced after stress or starvation, suggesting the presence of a promoter upstream of *ytkA* which is weakly cotranscribed with *dps.*

By using primer extension experiments, two putative 5′ ends of the message were mapped 19 and 32 bp upstream of the translational start point, suggesting that *dps* is transcribed monocistronically by two overlapping promoters, herein designated P_1 and P_B.

Both signals were seen in wild-type cells during exponential growth (Fig. 4B); however, the signal of the σ^B-dependent transcript was stronger than that of the σ^B-independent transcript. Transcription initiating from P_B was strongly increased in the wild type after heat, salt, and ethanol stress or starvation for glucose and was not detectable in a *sigB* null mutant. In contrast, transcription from the second promoter (P_1) decreased during the stress conditions in the wild type but was still present and weakly induced after heat shock in *sigB* mutant cells (Fig. 4B to C).

The σ^B-dependent 5′ end is preceded by the sequence 5′ GTTTTA-14 bp-GGGTAT 3′, which matches the consensus sequence for σ^B-dependent promoters that are found in the promoter regions of several other σ^B-dependent genes (22, 39). We interpret these results to indicate that during the general stress response, *dps* is transcribed by a σ^B-containing holoenzyme of RNA polymerase.

Dps is involved in nonspecific resistance to oxidative stress. Because in starved *E. coli* cells Dps is critical for the cells to survive toxic levels of H_2O_2 (1), we investigated the survival of

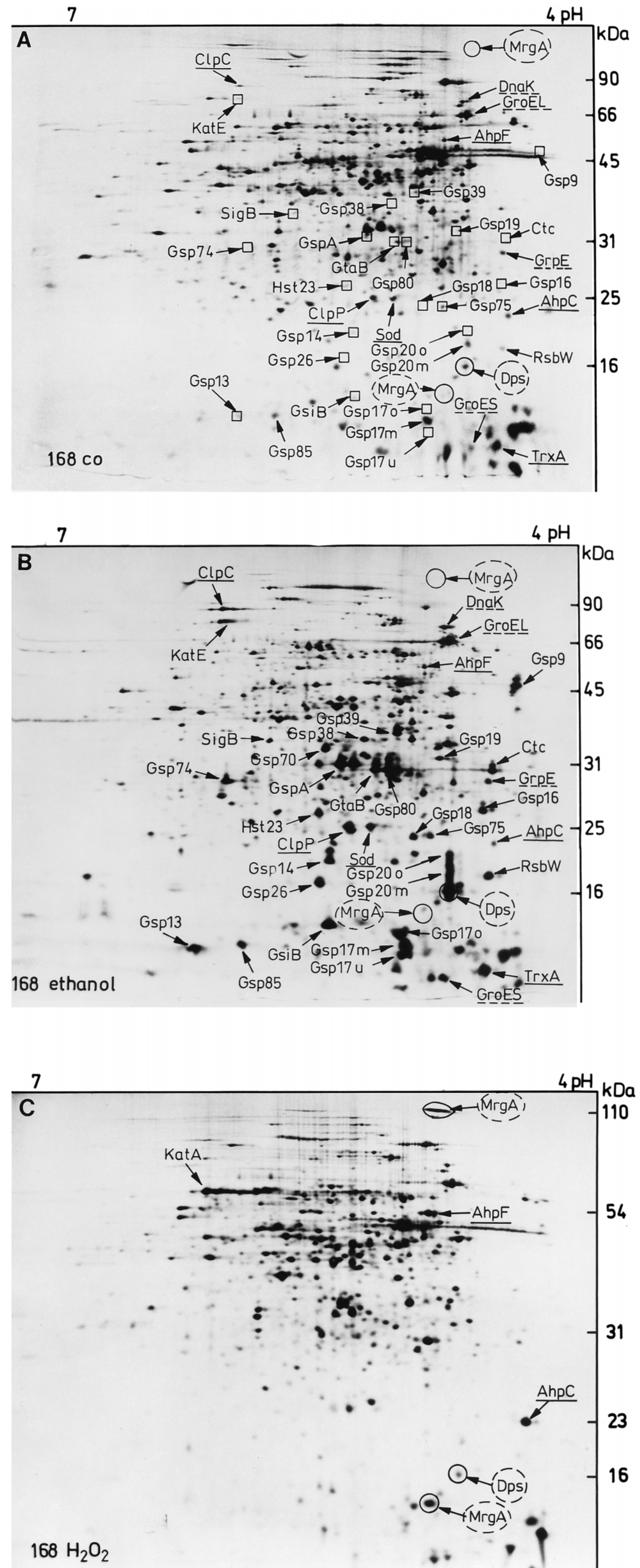

FIG. 3. Synthesis of stress proteins in *B. subtilis* wild-type cells labeled with L-[^{35}S]methionine before treatment (A) and 10 min after treatment with 4% ethanol (B) or 60 μM H_2O_2 (C). (A and B) Most of the σ^B-dependent Gsps, including Dps, are indicated. Gsps induced by a σ^B-independent mechanism are underlined, and heat-specific proteins are with dashes. (C) Peroxide-specific stress proteins, including MrgA, KatA, AhpC, and AhpF, are labeled. Dps and MrgA are marked by circles.

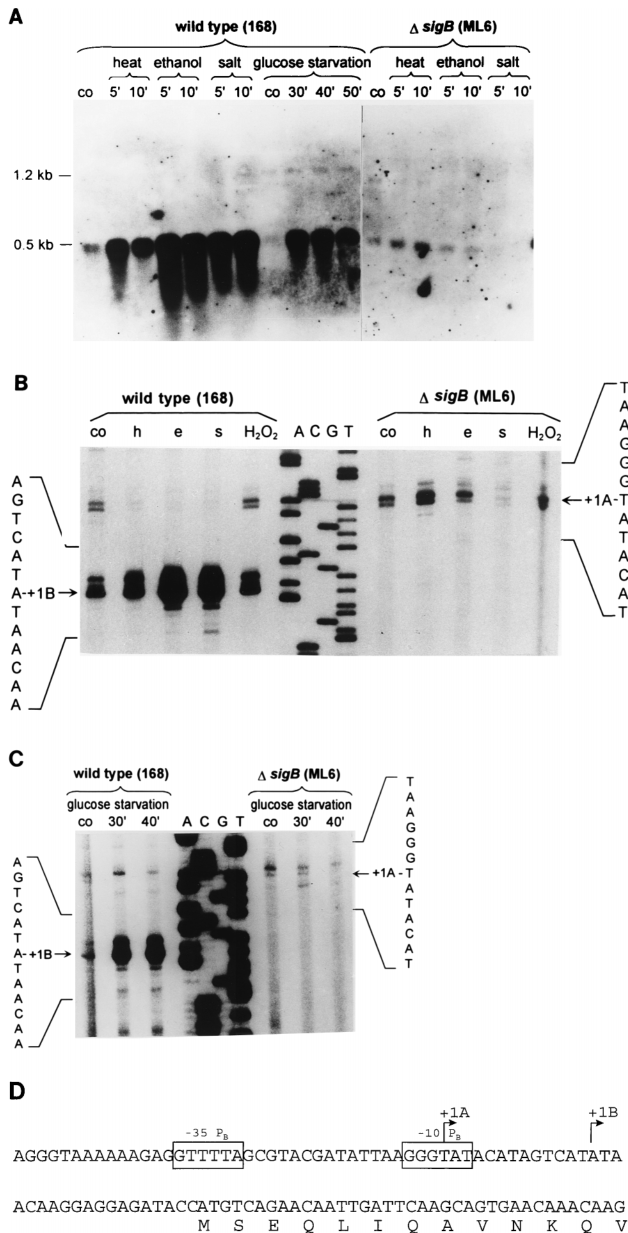

FIG. 4. Transcript analyses of *dps* and the sequence of the *dps* promoter region. For Northern blot (A) and primer extension (B and C) experiments, we added to each lane 10 μg of RNA isolated from either *B. subtilis* wild-type strain 168 or *sigB* mutant strain ML6 before (co) and at different times after exposure to heat (h), salt (s), ethanol (e), and oxidative stress or 30 and 40 min after the onset of the stationary phase provoked by starvation for glucose. The probable 5′ end of the σ^B-dependent *dps* message is indicated by +1B, and the probable 5′ end of the σ^B-independent *dps* transcript is indicated by +1A. The dideoxy sequencing ladder (ACGT) extends from the same primer used for primer extension and is complementary to that determined by DNA sequencing. (D) The transcriptional start points are marked by arrows, and the −10 and −35 promoter sequences of the σ^B-dependent transcript (P_B) are boxed. It is unknown if transcription of P_1 requires σ^A or another sigma factor. The N-terminal amino acid sequence of Dps is given below the nucleotide sequence.

a *dps* null mutant after oxidative challenge along the growth curve. The mutant showed a reduced rate of growth in minimal medium compared to that of the wild type (Fig. 5). During the exponential growth phase, both the wild type and the *dps* mutant rapidly lost viability after treatment with 10 mM H_2O_2. In contrast to the wild type, the *dps* mutant did not develop resistance to oxidative stress on entry into the stationary phase (Fig. 5). These results suggest that Dps is essential for the development of nonspecific stationary-phase resistance to oxidative stress.

Since *dps* mutant cells of *E. coli* showed a changed 2-D protein pattern (1), we compared the 2-D gels of ethanol-stressed *B. subtilis* wild-type cells and *dps* mutant cells labeled with [^{35}S]methionine. Spot Gsp20u, identified as Dps, was absent in Dps mutant cells, and furthermore, the spots designated Gsp20o and Gsp20m were not visible in the mutant (data not shown). No other differences in the patterns of general stress proteins synthesized in the wild-type and *dps* mutant strains could be detected (data not shown).

DISCUSSION

Growing cells are able to respond rapidly to specific growth-restricting conditions by the induction of stress-specific proteins to prevent cellular damage. However, bacteria spend most of their time in a nongrowing or nutrient-limited state which requires them to develop special strategies to ensure survival for longer periods, regardless of the nature of the growth-restricting factor. One of these nonspecific survival strategies of bacteria is the general stress response (22, 28, 31). More than 60 general stress proteins which are controlled by σ^B are induced after exposure to different types of stress or if cells enter the stationary phase. However, this suggested nonspecific protective function of the general stress proteins has remained a matter for speculation (9, 17, 24, 26). Very recently, we found that an essential function of some of these general stress proteins is to provide starving cells with nonspecific resistance to oxidative stress, which is developed after the onset of starvation (18). Surprisingly, KatE was not essential for this nonspecifically acquired resistance to oxidative stress (18). In this paper we identify one prominent Gsp as the Dps/PexB-homologous protein (1). We present data that Dps is essential for H_2O_2 resistance in glucose-starved *B. subtilis* cells. Therefore, the σ^B-dependent Dps of *B. subtilis* might have a function like that of the σ^S-dependent Dps protein of *E. coli*, which was shown to be involved in the nonspecific protection of DNA against oxidative damage under nutrient-limited conditions (1, 3, 29).

In *E. coli*, Dps is subject to complex regulation. During exponential growth, *dps* transcription is induced in an OxyR-dependent manner by sublethal doses of hydrogen peroxide. The same promoter is activated after the onset of the stationary phase or osmotic upshift by a σ^s-dependent mechanism. Therefore, Dps is part of the H_2O_2-inducible response as well as the general stress response in *E. coli* (3, 29).

In *B. subtilis* there are at least two Dps homologs, MrgA and Dps. Whereas MrgA is induced specifically after exposure to sublethal oxidative stress and, therefore, seems to be involved in the adaptive response to a lethal oxidative challenge (12, 13), the conditions which trigger the expression of *dps* in *B. subtilis* are rather nonspecific and require the presence of the general stress and starvation sigma factor, σ^B. Dps is strongly induced by exposure of exponentially growing cells to heat, salt, and ethanol stress or by starvation of stationary-phase cells for glucose. However, *dps* is very weakly transcribed even in a *sigB* deletion mutant from a promoter which overlaps the σ^B-dependent promoter region. Our results suggest that both promoters, which are separated by 12 bp, cannot be recognized at the same time after stress by the RNA polymerase. Only in the absence of σ^B is the weaker upstream σ^B-independent promoter utilized, indicating a P_1 promoter occlusion if the

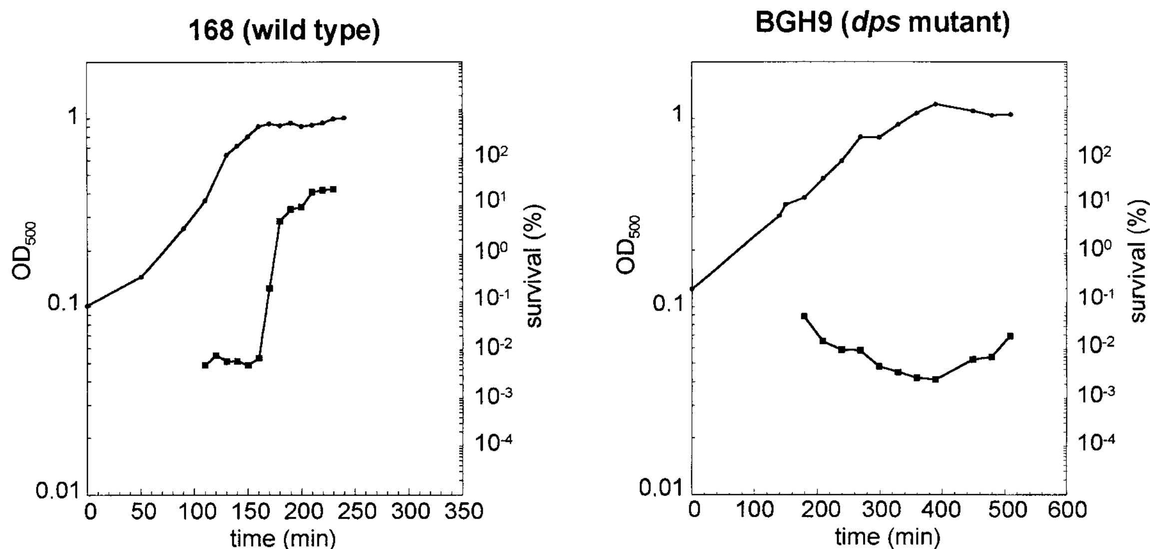

FIG. 5. A *dps* mutant strain failed to develop starvation-induced resistance to oxidative stress. Cultures grown under conditions of glucose starvation were challenged with 10 mM H_2O_2 for 10 min at the time points indicated and then plated on agar plates. Symbols: ●, culture density; ■, percent viability. Viability was determined by counting CFU. OD_{500}, optical density at 500 nm.

stronger σ^B-dependent promoter is engaged in transcriptional initiation.

The presence of two Dps homologs in *B. subtilis* controlled by different mechanisms enables the cell strictly to differentiate between the specific and general stress responses. The same regulatory phenomenon was found for the catalases KatE and KatA of *B. subtilis*. Whereas the vegetative catalase KatA specifically confers adaptive resistance to oxidative stress to exponentially growing cells, the other catalase, KatE, was nonspecifically induced in response to heat, salt, and ethanol stress or in response to starvation for glucose (18, 19).

In addition to being expressed after oxidative stress, MrgA is also expressed at the onset of the stationary phase provoked by limitation for iron and manganese. Therefore, it might be possible that MrgA complements the loss of resistance to starvation-induced oxidative stress in *dps* mutant cells if cells are grown with limiting amounts of iron and manganese. However, survival experiments under conditions of metal ion limitation showed the same results as those of experiments using large amounts of metal ions (data not shown). This indicates that Dps is essential for H_2O_2 resistance in stationary-phase *B. subtilis* cells and that the loss of Dps may not be compensated by MrgA.

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