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Forespore-Specific Expression of *Bacillus subtilis yqfS*, Which Encodes Type IV Apurinic/Apyrimidinic Endonuclease, a Component of the Base Excision Repair Pathway

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The temporal and spatial expression of the *yqfS* gene of *Bacillus subtilis*, which encodes a type IV apurinic/apyrimidinic endonuclease, was studied. A reporter gene fusion to the *yqfS* opening reading frame revealed that this gene is not transcribed during vegetative growth but is transcribed during the last steps of the sporulation process and is localized to the developing forespore compartment. In agreement with these results, *yqfS* mRNAs were mainly detected by both Northern blotting and reverse transcription-PCR, during the last steps of sporulation. The expression pattern of the *yqfS-lacZ* fusion suggested that *yqfS* may be an additional member of the Eσ^G regulon. A primer extension product mapped the transcriptional start site of *yqfS*, 54 to 55 bp upstream of translation start codon of *yqfS*. Such an extension product was obtained from RNA samples of sporulating cells but not from those of vegetatively growing cells. Inspection of the nucleotide sequence lying upstream of the in vivo-mapped transcriptional *yqfS* start site revealed the presence of a sequence with good homology to promoters preceding genes of the σ^G regulon. Although *yqfS* expression was temporally regulated, neither oxidative damage (after either treatment with paraquat or hydrogen peroxide) nor mitomycin C treatment induced the transcription of this gene.

Endogenous and environmental factors such as reactive oxygen species, UV light, and chemical carcinogens alter the chemical structure of DNA bases, producing lesions that are substrates for a myriad of DNA glycosylases of the base excision repair (BER) pathway (27). The apurinic/apyrimidinic (AP) sites generated not only by the action of DNA glycosylases but also by the spontaneous depurination and depyrimidination of DNA (29, 30) are inherently toxic and highly mutagenic and thus should be rapidly processed and eliminated (31). The first catalytic event during the repair of AP sites is carried out by AP endonucleases, which cleave the DNA backbone immediately 5′ of an AP site, generating a 5′ deoxyribose-phosphate group and a 3′ deoxyribose-hydroxyl group. AP endonucleases have been classified into two families, namely, ExoIII and type IV AP endonucleases (3, 13), and these enzymes have been conserved across the species of the three domains of life (23).

Dormant spores of *Bacillus subtilis* are more resistant than their vegetatively growing counterparts to several chemical substances, including acids, bases, alkylating agents, and oxidizing agents (reviewed in references 40, 41, and 58). The existence of core coats, the low permeability of spores to hydrophilic compounds, and the protection of spore DNA from damage by its saturation with α/β-type small acid-soluble proteins (SASPs) account for this resistance (reviewed in references 40, 56, and 58). It has been demonstrated that α/β-type SASPs slow DNA depurination-depyrimidination, as well as hydroxyl radical-induced DNA backbone cleavage, thus contributing to spore resistance to heat and oxidizing agents (reviewed in references 40 and 58). α/β-type SASPs bind to spore DNA and are in part responsible of the strong resistance of *B. subtilis* spores to UV light (reviewed in references 40, 41, and 58); however, these DNA-binding proteins do not confer protection to DNA against base alkylation (55).

The genome of *B. subtilis* (26) possesses genes that potentially encode ExoIII and type IV AP endonucleases, namely, *exoA* and *yqfS*, whose products show a high level of homology to ExoIII and type IV AP endonucleases, respectively. Although the enzymology of *B. subtilis* ExoA has been studied in detail (53), nothing has been reported regarding the mechanisms that control its expression during growth and sporulation of *B. subtilis*.

The expression of DNA repair systems in the gram-positive spore-forming bacterium *B. subtilis* has been shown to be differentially regulated during growth and differentiation (4, 11, 32, 34), as well as during spore germination and outgrowth (54). DNA lesions acquired during unpredictable periods of *B. subtilis* spores dormancy must be necessarily corrected during germination by spore-specific expressed DNA repair systems (reviewed in references 40 and 58). The best example studied thus far is the correction of the UV-C induced spore photoproduct (5-thyminil-5,6-dihydrothymine) through both the specific spore photoproduct lyase protein (SplB) and the general excision-repair system (UVR) (reviewed in references 40 and 41). However, during unpredicted periods of spore dormancy *B. subtilis* spores could potentially accumulate, in addition to spore photoproduct (SP), different types of DNA lesions, such as strand brakes, cyclobutane pyrimidine dimers (CPDs), chemically altered bases, and AP sites that could affect essential functions such as transcription and replication during ger-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype[a]	Source (reference)
Strains		
B. subtilis		
168	*trpC2*	Laboratory stock
WN118	*sigGΔ1 trpC2*	Wayne Nicholson
PERM317	*trpC2 yqfS-lacZ;* Cm^r	This study
PERM336	*sigGΔ1 trpC2 yqf*S-*lacZ*	This study
YB3000	*metB5 trpC2 xin- 1 sigB amyE* (deleted for spβ) pCCR202 (*recA-lacZ* at *amyE*); Cm^r	R. E. Yasbin
E. coli		
SURE	e14$^-$(McrA$^-$) Δ(*mcrCB-hsdSMR-mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5* (Kan^r) *uvrC* [F′ *proAB lacI^q lacZ*ΔM15 Tn*10* (Tet^r)]	Stratagene
PERM162	*E. coli* SURE, pUC18; Amp^r Tc^r	This study
PERM253	*E. coli* SURE, pPERM253; Amp^r Tc^r	This study
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*/F′ *proAB lacI^q*ZΔ M15 Tn*10* (Tet^r)	Stratagene
PERM267	*E. coli* XL1-Blue, pPERM253; Amp^r	This study
XL10-Gold Kan	Tet^r Δ(*mcrA*) *183,* Δ(*mcrCB-hsdSMR-mrr*); *173 endA1 sup E44 thi-1 recA1 gyrA96 relA1 lacHte* [F′ *proAB lacI^{q}Z*ΔM15 Tn*10* (Tet^r) Tn*5* (Kan^r) Amy]	Stratagene
PERM348	*E. coli* XL-Gold, pPERM348, Amp^r	This study
Plasmids		
pJF751	Integrational *lacZ* fusion vector; Cm^r	W. Nicholson (17)
pUC18	Multisite *E. coli* cloning vector	Laboratory stock (63)
pPERM253	*yqfS* gene cloned in pUC18	This study
pPERM267	514-pb *Eco*RI/*Nae*I fragment of *yqfS* from pPERM253 cloned in pJF751	This study
pPERM348	*yqfS* ORF cloned into the *Bam*HI site of PQE-30	This study

[a] Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Tc^r, tetracycline resistant.

mination (40). Although the expression of *splB* in the forespore compartment by σ^G RNA polymerase has been widely substantiated (44, 45), few data exist in the literature concerning the expression of other specific or general DNA repair systems in the forespore compartment.

As mentioned above, in the genome of *B. subtilis* exists an open reading frame (ORF), *yqfS*, whose predicted product shows 53% homology with the type IV AP endonuclease of *Escherichia coli.* We describe here the expression of the cloned *yqfS* gene of *B. subtilis* from an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter in *E. coli.* Our results demonstrate that a His_6-YqfS purified enzyme is able to process the cleavage of abasic sites in the DNA. In addition, our results demonstrated that the expression of *yqfS* is forespore specific but was not induced by the stress imposed by superoxide radicals, by hydrogen peroxide, or by the DNA-damaging agent mitomycin C.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *B. subtilis* and *E. coli* strains used in the present study are shown in Table 1. Plasmids used in this work are listed in Table 1. Media used were Difco sporulation medium (DSM) (52) and Luria-Bertani (LB) medium (38). When appropriate, antibiotics were added to the medium at the following final concentrations: chloramphenicol, 3 μg/ml; ampicillin, 50 μg/ml; and kanamycin, 10 μg/ml. Liquid cultures were shaken at 250 rpm at 37°C. Cultures on solid medium were grown at 37°C. The optical density (OD) of liquid cultures was monitored with a Pharmacia Ultrospec 2000 spectrophotometer set at 600 nm.

Genetic and molecular biology techniques. Preparation of competent *E. coli* or *B. subtilis* cells and their transformation with DNA was performed as described elsewhere (5, 49). Extraction of chromosomal DNA from *B. subtilis* was carried out according to the protocol of Cutting and Vander Horn (12). Small-scale preparation of plasmid DNA from *E. coli* cells, enzymatic manipulations, and agarose gel electrophoresis were performed by standard techniques (49). Large-scale preparation and purification of plasmid DNA was accomplished by using commercial ion-exchange columns according to the instructions of the supplier (Qiagen, Inc., Valencia, Calif.). Nucleic acid sequencing by dideoxynucleotide chain termination (50) was performed with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (U.S. Biochemical Corporation, Cleveland, Ohio). Sequencing products were analyzed by autoradiography after electrophoresis through a 6% polyacrylamide sequencing gel. Alternatively, DNA plasmids purified through Qiagen columns were processed for sequencing in a Perkin-Elmer (Norwalk, Conn.) model 377A automated DNA sequencer.

Cloning of *yqfS* and construction and integration of a *yqfS-lacZ* gene fusion. The complete *yqfS* gene was amplified by PCR with genomic DNA from *B. subtilis* 168 as a template and the oligonucleotide primers 5′-GGGAATTCGCCGAAGAAGGTTAAGCC-3′ (forward) and 5′-CGGGATCCGGCCGTTGAAGTAGCGAACC-3′ (reverse). The primers were designed to insert *Eco*RI and *Bam*HI sites (underlined). Amplification was performed on 0.1 μg of chromosomal DNA by using an MJ Research (Watertown, Mass.) Minicycler with *Vent* DNA polymerase (New England Biolabs, Beverly, Mass.) according to the manufacturer's recommendations. The 1,181-bp PCR fragment extending from 110 bp upstream of the *yqfS* start codon through 157 bp downstream of the *yqfS* stop codon was digested with *Sma*I and *Bam*HI and ligated into pUC18 to generate pPERM253. pPERM253 was replicated in *E. coli* XL1-Blue, and the cloned *yqfS* gene was sequenced on both strands.

Construction of an in-frame translational *yqfS-lacZ* fusion was performed in the integrative plasmid pJF751 (17) by inserting a 472-bp *Eco*RI-*Nae*I fragment from plamid pPERM253 into pJF751 previously digested with *Eco*RI and *Sma*I. The resulting construction, containing the *yqfS-lacZ* fusion and designated pPERM317, was propagated into *E. coli* XL1-Blue. Plasmid pPERM317 was introduced by transformation into competent cells of *B. subtilis* 168, and transformants were selected on solid DSM containing chloramphenicol.

Purification of His_6-YqfS and substrates for AP endonuclease activity. *E. coli* PERM348 containing plasmid pPERM348 (Table 1) was grown in 50 ml of LB medium, supplemented with ampicillin (100 μg/ml), at 37 °C to an OD at 600 nm (OD_{600}) of 0.5. Expression of *yqfS* was induced during 4 h at 37°C by the addition of IPTG to 0.5 mM. Cells were collected by centrifugation and washed two times with 10 ml of 50 mM Tris-HCl (pH 7.5)–300 mM NaCl (buffer A). The cells were disrupted in 10 ml of the same buffer containing lysozyme (10 mg/ml) for 30 min at 37°C. The cell homogenate was subjected to centrifugation to eliminate undisrupted cells and cell debris, and the supernatant was applied to a 5-ml nickel-nitrilotriacetic acid-agarose column previously equilibrated with buffer A. The column was washed with 50 ml of buffer A containing 10 mM imidazole plus 50

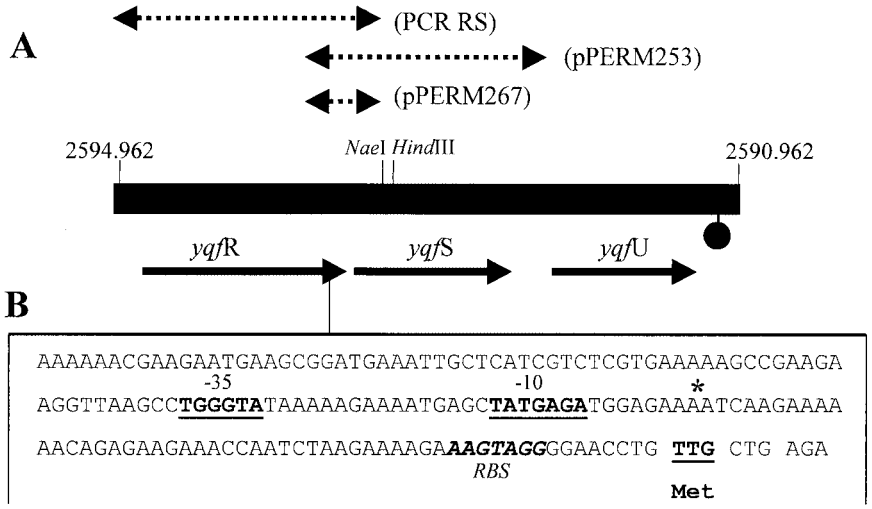

FIG. 1. *yqfS* region of the *B. subtilis* chromosome and DNA sequences lying upstream of the *yqfS* ORF. (A) Genetic organization of the *yqfS* locus between indicated coordinates of the *B. subtilis* chromosome (filled box). Dashed lines above the ORFs (arrows) show the DNA fragments cloned into the indicated plasmids. Downstream of *yqfU* ORF a putative transcriptional terminator is shown (stem-loop structure). (B) Sequence of the intergenic region between *yqfR* and *yqfS*. The in vivo-mapped transcriptional start site of *yqfS* is indicated by an asterisk immediately downstream of the −10 and −35 sequences that might function as a promoter for RNA polymerase-σ^G. *RBS* (putative ribosome-binding site).

ml of buffer A containing 20 mM imidazole, and the protein bound to the resin was eluted with 15 ml of buffer A containing 100 mM imidazole; 2-ml fractions were collected during this last step. Aliquots (15 μl) of the cell homogenate, the flowthrough, and the bound fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Two types of substrates were prepared to assay AP endonuclease activity of His_6-YqfS, namely, pBluescript (Stratagene), which was partially depurinated after a previously described protocol (28) and a 5′-end-radiolabeled double-stranded 19-mer nucleotide containing a single abasic site (20).

The endonuclease activity of His_6-YqfS against pBluescript containing AP sites (AP-pB) was determined in a mixture reaction of 25 μl containing 600 ng of purified His_6-YqfS and 100 ng of substrate in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol. The reactions were incubated at 37°C for 30 min and analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Endonuclease activity against the double-stranded radiolabeled 19-mer containing a single AP site was performed in a total volume of 15 μl containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 500 nmol of unlabeled and 10 nmol of double-stranded radiolabeled 19-mer containing a single AP site. Different amounts of His_6-YqfS were added to the mixture reactions and incubated for 30 min at 37°C. The reactions were separated on a 20% denaturing acrylamide gel and then subjected to autoradiography.

Cell growth and enzymatic assays. *B. subtilis* strains carrying the *yqfS-lacZ* fusion were grown and allowed to sporulate in liquid DSM. Samples of 1.5 ml were collected during vegetative growth and throughout sporulation. Cells were washed with 0.1 M Tris-HCl (pH 7.5) and processed for determination of β-galactosidase (42) and glucose dehydrogenase (GDH) activities (19, 42). The β-galactosidase activities were determined in cell extracts obtained from mother cells and forespore fractions prepared according to a previously described protocol (36, 44).

Northern blot and primer extension experiments. The total RNA for both Northern blotting experiments and mapping of the 5′end of *yqfS* was isolated as previously described (35). Northern blots were performed with RNA samples isolated from strains *B. subtilis* 168 and WN118 (*sigG* mutant). RNA samples (20 μg) were separated by electrophoresis through 1% agarose-formamide gel and transferred to a high-bound nylon membrane. The membrane containing the transferred RNA was hybridized at 70°C with a 1,181-pb *Eco*RI-*Bam*HI fragment from pPERM253 containing the entire *yqfS* sequence. The probe was labeled by random priming with [α-^{32}P]dCTP by using the Rediprime II DNA labeling system according to the instructions of the provider (Amersham Biosciences, Buckinghamshire, England). Detection of hybrids was performed by autoradiography exposing the membrane to Kodak X-Omat films.

The 5′ end of *yqfS* was mapped by primer extension (37) of *yqfS* transcripts produced during sporulation. To this end, total RNA was isolated from vegetative and sporulating cells of *B. subtilis* PERM317. In order to obtain the maximum amount of *yqfS* transcripts during sporulation, we monitored the expression of β-galactosidase activity directed by the *yqfS-lacZ* fusion in this strain. The total RNA (40 μg from each sample) was hybridized with the 20-mer oligonucleotide 5′-CGGCGCGTATTTTGCGGTGC-3′, which was complementary to the *yqfS* mRNA from nucleotides 106 to 124 downstream from the putative *yqfS* translational start codon. The oligonucleotide was labeled on its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. The primer was extended with Moloney murine leukemia virus reverse transcriptase, and the extended products were separated by electrophoresis through a 6% polyacrylamide DNA sequencing gel. The position of the extended products was determined by running a sequencing reaction generated with the same 20-base primer and a 1,978-bp PCR product (PCR RS) extending from 247 bp upstream of the *yqf*R start codon to 416 bp downstream of the start codon of *yqfS* (Fig. 1).

RT-PCR experiments. Total RNA from vegetative or sporulating *B. subtilis* 168 cells, grown in DSM, was isolated by using the TRI reagent (Molecular Research Center, Inc.). Reverse transcription-PCRs (RT-PCRs) were performed with the RNA samples and a Master Amp RT-PCR kit (Epicentre Technologies) according to the instructions of the provider. The primers used for RT-PCRs were 5′-CCTGTTGCTGAGAATAGGC-3′ (forward) and 5′-CGGCGCGTAT TTTGCGGTGG-3′ (reverse) to generate a 132-bp RT-PCR product extending from 4 bp upstream from the start codon of *yqfS* to 128 bp downstream of this point (Fig. 1). As a control, in each experiment, the absence of chromosomal DNA in the RNA samples was assessed by mounting PCRs with *Vent* DNA polymerase (New England Biolabs) and the set of primers described above.

RESULTS

Cloning of *yqfS*. The existence of a type IV AP endonuclease in the genome of *B. subtilis* was investigated by using the primary structure of *E. coli* Nfo (51) as a query to search against the database of National Center for Biotechnology Information with a Gapped BLAST program (2). As described in Materials and Methods, this approach was used to retrieve a gene termed *yqfS* from the genome of *B. subtilis* (26). Analysis of the *yqfS* primary structure revealed an ORF of 891 bp with enough information for the synthesis of a predicted protein of 31 kDa. Amino acid alignments showed that YqfS possesses homologies of 53, 52, and 32% with *E. coli* Nfo (51),

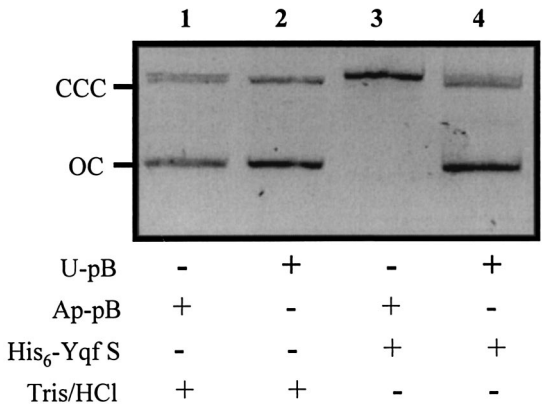

FIG. 2. Endonuclease activity of His_6-YqfS against a plasmid containing AP sites. Aliquots (600 ng) of His_6-YqfS were incubated with 100 ng of either untreated (U-pB [lane 4]) or AP-containing sites (AP-pB [lane 3]) of pBluescript. Lane 1, AP sites-containing plasmid incubated with 50 mM Tris-HCl (pH 7.5)–300 mM NaCl; lane 2, untreated plasmid incubated with 50 mM Tris-HCl (pH 7.5)–300 mM NaCl. The reactions were incubated at 37°C for 30 min and then analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

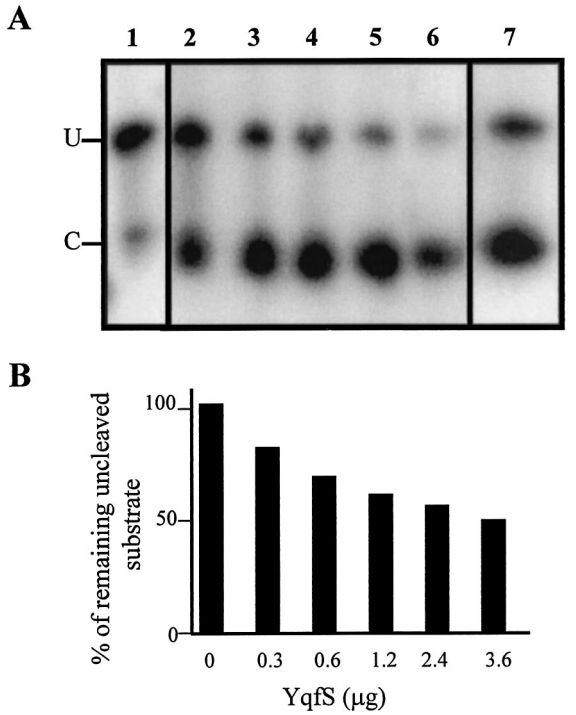

FIG. 3. Endonuclease activity of His_6-YqfS against a double-stranded 19-mer containing a single AP site. (A) A total of 510 nmol of 5′-end-radiolabeled double-stranded 19-mer nucleotide containing a single AP site was incubated for 30 min at 37°C with different concentrations of His_6-YqfS. The reactions were separated on a 20% denaturing acrylamide gel and then subjected to autoradiography. Lane 1, no enzyme; lanes 2 to 6, 0.3, 0.6, 1.2, 2.4, and 3.6 μg of His_6-YqfS, respectively; lane 7, 2 U of *E. coli* Nfo. Radioactively labeled cleaved (C) and uncleaved (U) strands are as indicated. (B) Densitometry of the experiment shown in panel A; the percentage of uncleaved substrate was plotted as a function of the amount of His_6-YqfS added to the reaction.

Saccharomyces cerevisiae Apn1 (46), and *Thermotoga maritima* endonuclease IV (20), respectively.

Purification and enzymatic activity of YqfS. The His_6-YqfS protein synthesized in *E. coli* was purified to homogeneity by metal chelate affinity chromatography, yielding a 36-kDa protein (data not shown).

To corroborate the predicted AP endonuclease activity of YqfS, two enzymatic assays were performed. First, the His_6-YqfS pure enzyme was incubated with a partially depurinated plasmid DNA as a substrate (AP-pB). The results presented in Fig. 2 reveal the conversion of the closed covalently circular depurinated plasmid (CCC) to the open circular form (OC) due to single-strand breaks performed by the His_6-YqfS purified protein (lane 3). As shown in Fig. 2 (lane 4), the nondepurinated plasmid (U-pB) was not a substrate for the His-tagged YqfS protein. Controls shown in Fig. 2 revealed that neither the untreated nor the depurinated plasmid were converted to the OC form in the absence of the His6-YqfS protein (lanes 1 to 2). Second, a 5′-end radiolabeled double-stranded 19-mer nucleotide containing a single AP site was used as a substrate for the YqfS pure protein. Essentially, different amounts of the His_6-tagged protein were incubated with 510 nM of this AP substrate. The products of the reaction analyzed on a denaturing polyacrylamide gel revealed that the endonucleolytic activity of YqfS at the AP site was dependent on the concentration of the enzyme used (Fig. 3A, lanes 2 to 6). To better evaluate this conclusion, these results were analyzed by densitometry, thereby corroborating that cleavage of the AP substrate by His_6-YqfS is concentration dependent (Fig. 3B). Although the radiolabeled 20-bp-mer was also cleaved by *E. coli* Nfo (Fig. 3A, lane 7), it was observed that a fraction of the substrate was partially degraded (Fig. 3A, lane 1). The results presented in Fig. 3A (lane 7) also revealed that another fraction of the radiolabeled substrate was inaccessible to the enzyme; such a fraction most probably corresponded to nondepurinated compound. These results demonstrate for the first time that the product encoded by the *yqfS* gene possesses activity of AP endonuclease, a result in agreement with its high structural similarity to the family IV AP endonucleases.

Expression of a *yqfS* during growth and sporulation. The strain *B. subtilis* PERM317 harboring a single copy of the *yqfS-lacZ* fusion was grown in DSM to induce sporulation. Determination of *yqfS*-directed β-galactosidase activity during growth and sporulation stages revealed a temporal pattern of expression. Although no β-galactosidase activity was detected during vegetative growth, Fig. 4 reveals that enzymatic activity was detectable after T_0, reached a maximum during T_6 and T_7, and then decreased. The expression pattern of the reporter gene (Fig. 4) was similar to that observed for genes whose expression occurs during the last steps of sporulation in the forespore compartment, such as the operon *splA-splB* (44), *gdh* (39), and *ssp* (35, 36) genes. To further investigate this observation, two approaches were followed. First, cell fractioning experiments were performed to investigate whether the expression of the reporter gene occurred inside of the spore. The results of Fig. 4 show that β-galactosidase activity started to accumulate inside of the forespores from sporulation stage T_5 and continued to accumulate until at least stage T_9.

The cell extracts used to determine β-galactosidase activity

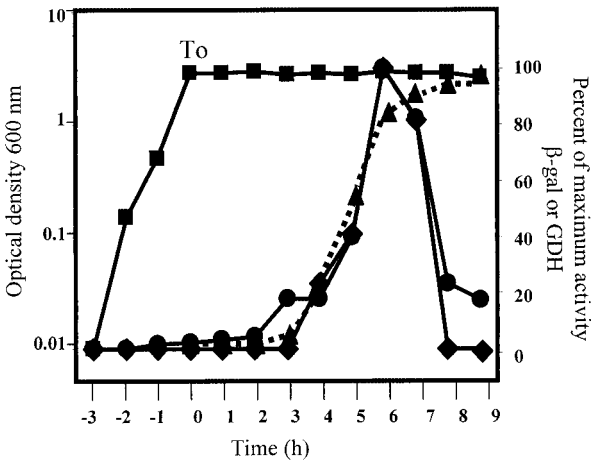

FIG. 4. Expression of a *yqfS-lacZ* translational fusion during growth and sporulation of *B. subtilis*. *B. subtilis* PERM317 was grown to sporulation in liquid DSM (■). Samples were collected at different times and treated with lysozyme, and the extracts were assayed for either β-galactosidase (♦) or GDH (●) activity. The β-galactosidase activity inside of the forespore lysozyme-resistant fraction (▲) was assayed as described in Materials and Methods.

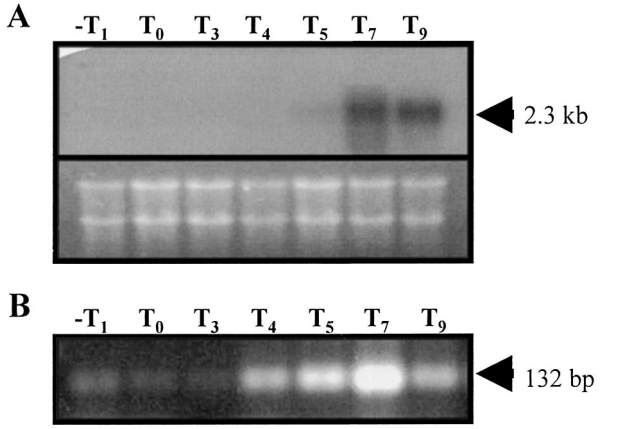

FIG. 5. Northern blot (A) and RT-PCR analysis (B) of *yqfS* transcription during vegetative growth and sporulation. (A) *B. subtilis* 168 was induced to sporulate in liquid DSM. Total RNA was isolated (35) during the times (in hours) indicated (T_0 = end of exponential growth). Then, 20-μg samples were separated on agarose-formaldehyde gels (lower panel) and transferred to nylon membranes. The membrane was hybridized with a ^{32}P-labeled 1,181-bp fragment encompassing the entire *yqfS* sequence as described in Materials and Methods. (B) RNA samples (1 μg) isolated from a *B. subtilis* 168 DSM culture at the times indicated (in hours) were processed for RT-PCR analysis as described in Materials and Methods. The arrowhead shows the size of the expected RT-PCR product.

were also assayed for GDH activity, an enzyme encoded by the stage III, forespore-specific *gdh* gene (19, 39). The results shown in Fig. 4 revealed that the expression patterns of the *yqfS-lacZ* fusion and the GDH activity followed essentially identical kinetics, strongly indicating that *yqfS* gene expression is activated in the forespore compartment during the last steps of sporulation.

To further support this contention, Northern blot experiments were performed with total RNA isolated from cells of strain *B. subtilis* PERM168 collected before and after the onset of sporulation. The results (Fig. 5A) indicated that *yqfS* mRNA appeared as a 2.3-kb band during sporulation stages T_5 through T_9, observing a major hybridization signal at T_7. As shown in Fig. 5A, no signal was detected in the blot with RNA isolated from cells growing exponentially, supporting the conclusion that *yqfS* expression is sporulation specific. Moreover, RT-PCR experiments resulted in the major amplification of a *yqfS* product when total RNA isolated from sporulating cells was used as a template. Figure 5B shows that the RT-PCR product of *yqfS* (132 bp) was more abundant with RNA samples of the step T_7 of sporulation.

σ^G dependence of *yqfS* expression. The expression of forespore specific genes in *B. subtilis* is carried out through the sequential action of two temporally expressed RNA polymerases containing either σ^F or σ^G factors (21, 43). However, as shown above, the expression pattern of the *yqfS-lacZ* fusion was very similar to the σ^G-dependent *gdh* gene, suggesting that *yqfS* is under the control of σ^G-containing RNA polymerase. This notion was directly tested by two different approaches. First, the *yqfS-lacZ* fusion was introduced by transformation into competent cells of *B. subtilis* WN126 harboring a deletion of the σ^G gene, an *spo* mutant in which sporulation is arrested during stage III (24, 60). The resulting strain, *B. subtilis* PERM336, grown in DSM expressed very low levels of *yqfS*-directed β-galactosidase activity during both vegetative- and stationary-growth phases (data not shown). Consistent with this result, the levels of GDH in this strain were almost zero (data not shown).

In a second approach, Northern blot experiments were performed with RNA isolated from vegetative and stationary cells of *B. subtilis sigGΔ1* grown in liquid DSM. The results shown in Fig. 6A revealed the lack of *yqfS* mRNAs in this *sigG* mutant genetic background, since no hybridization signal was detected during both exponential-growth-phase and stationary-growth-phase cells. Such a result was also confirmed by RT-PCR experiments, which failed to amplify the 132-bp *yqfS* fragment from RNA samples isolated before and after the onset of sporulation (Fig. 6B). Taken together, these results are consistent with *yqfS* expression being dependent on σ^G RNA polymerase.

Mapping the transcriptional start site of *yqfS*. The genetic organization of the *yqfS* locus reveals that this gene is flanked upstream by *yqfR*, which encodes a putative RNA helicase, and downstream by *yqfU*, which encodes a protein of unknown function (Fig. 1). The existence of only one potential transcriptional terminator until the end of *yqfU* suggests that the three genes could be cotranscribed as a polycistronic message. To investigate this possibility, primer extension analysis was performed to map the 5′ ends of the mRNAs originating from upstream from the *yqfS* coding sequence. Experiments were carried out with total RNA isolated from *B. subtilis* PERM317 harboring the *yqfS-lacZ* fusion. Cells used to isolate RNA were harvested during both vegetative growth and the T_7 sporulation stage, the time of maximum expression of the *yqfS-lacZ* fusion. The results shown in Fig. 7 (lane 2) revealed the synthesis of a major extension product located 54 to 55 bp upstream of translation start codon of *yqfS*. Such an extension product was obtained only in experiments performed with

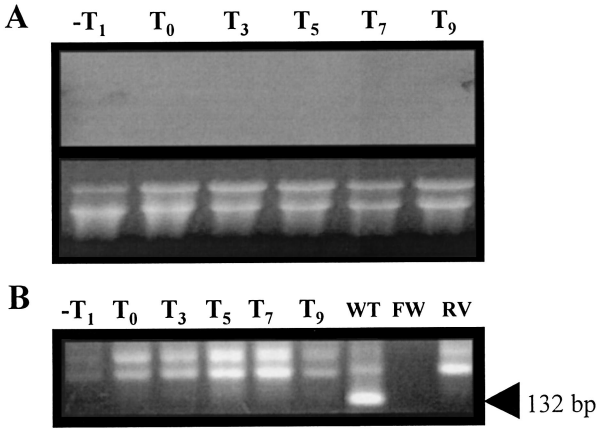

FIG. 6. Northern blot (A) and RT-PCR analysis (B) of *yqfS* transcription during vegetative growth and sporulation of *B. subtilis sigGΔ1* (strain WN118). (A) *B. subtilis* WN118 was grown in liquid DSM. Total RNA was isolated (35) during the times indicated (in hours). Samples (20 μg) were separated on agarose-formaldehyde gels (lower panel) and transferred to nylon membranes. The membrane was hybridized with a ^{32}P-labeled 1,181-bp fragment encompassing the entire *yqfS* sequence as described in Materials and Methods. (B) RNA samples (1 μg) isolated at the times indicated (in hours) from a *B. subtilis sigGΔ1* DSM culture were processed for RT-PCR analysis as described in Materials and Methods. For the wild type (WT), the RNA was isolated from *B. subtilis* 168 (Fig. 5); FW was obtained with the forward primer in the absence of RNA, and RV was obtained with the reverse primer in the absence of RNA. The arrowhead shows the size of the expected RT-PCR product.

RNA isolated from sporulating cells but not with RNA of vegetatively growing cells (Fig. 7, lane 1). Inspection of the nucleotide sequences lying upstream of the in vivo mapped transcriptional *yqfS* start site revealed the existence of sequences with good homology to promoters preceding genes of the σ^G regulon (Fig. 8) (21, 43). A higher level of homology was found in the −35 region, which possessed three of the four

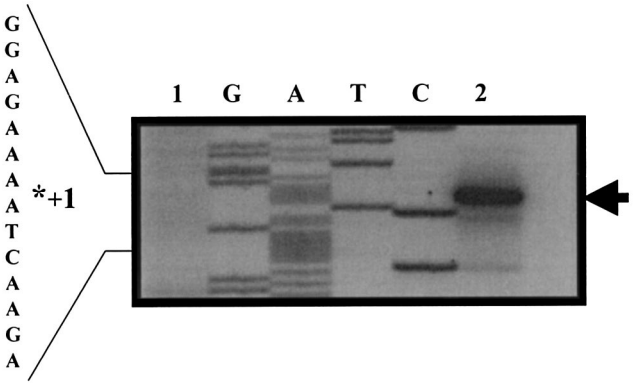

FIG. 7. Primer extension analysis for mapping the transcriptional start site of *yqfS*. Total RNA was isolated (34) from either vegetative (lane 1) or sporulating (stage T_7; lane 2) *B. subtilis* PERM317 cells grown in DSM. Primer extension was performed as described in Materials and Methods. The asterisk indicates the position of the primer extension product in the DNA sequence lying upstream of *yqfS* (see Fig. 1). The 5′ end of the *yqfS* transcript was determined by running a DNA sequencing ladder generated with the same primer (lanes G, A, T, and C) and was labeled with an arrowhead.

P*sigG*: TGA**ATA**-NNNNNNNNNNNNNNNN-**CAT**AC**TA**

*

P*yqfS*: TGGG**TA**-TAAAAAGAAAATGAGC-T**AT**GAG**A**TGGAGAAAA

FIG. 8. Comparison of the consensus Eσ^G (19) promoter sequence (top line) with the putative promoter sequence lying upstream of *yqfS* (bottom line). Absolutely conserved (boldface) or highly conserved (underlined) bases in Eσ^G-type promoters (21, 43). The position of the mapped transcriptional start site of *yqfS* is indicated with an asterisk.

absolutely conserved bases present on *sigG* promoters (Fig. 8). On the other hand, the −10 region conserved three of the four absolutely conserved residues observed in such σ^G promoters. However, it was found that the −10 and −35 regions were separated by 16 bp instead of the reported 17 to 18 bp for the σ^G consensus sequence (Fig. 8).

Induction of the *yqfS-lacZ* fusion by oxidative stress or during the SOS response. In *E. coli*, the expression of the type IV AP endonuclease *nfo* gene is induced by generators of superoxide radicals, such as paraquat (8). On the other hand, *B. subtilis* responds to H_2O_2 stress displaying an adaptive response that induces the expression of genes such as *katA* (catalase), *ahpCF* (alkyl hydroperoxide reductase), *mrgA*, and the *hemA* operon (1, 4, 7, 9, 10, 14). We therefore investigated whether the *yqfS* gene in *B. subtilis* is also induced by the oxidative stress imposed by either superoxide radicals or hydrogen peroxide. To this end, the strain *B. subtilis* PERM317 containing the *yqfS-lacZ* fusion, integrated into the *yqfS* locus, was grown in LB medium to the mid-exponential phase and treated with paraquat (10 μM) or hydrogen peroxide (200 μM). The results (Fig. 9A) revealed that, at the concentrations

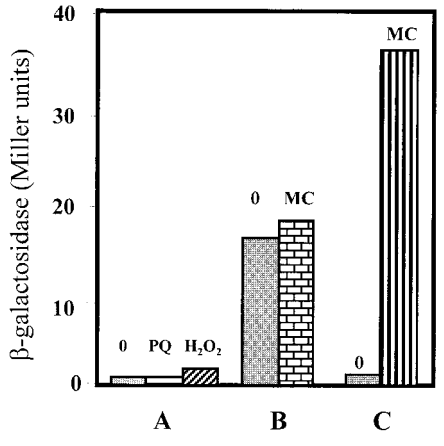

FIG. 9. Lack of induction of a *yqfS-lacZ* fusion by paraquat, H_2O_2, or mitomycin. *B. subtilis* PERM317 was grown to an OD_{600} of 0.5 in either minimal Spizizen medium (A) or LB medium (B). The culture made in minimal Spizizen medium was divided into three subcultures; one (labeled "0") was left untreated, and the other two were treated with either paraquat (PQ; 10 μM) or H_2O_2 (200 μM). The LB culture was treated in the same manner except that mitomycin C (MC; 0.5 μg/ml) was added to the culture. (C) *B. subtilis* YB3000 was grown in LB medium to an OD_{600} of 0.5; at this point, the culture was equally divided, and mitomycin C (0.5 μg/ml) was added to one of the subcultures. In all cases, the β-galactosidase activity was determined with cell samples collected 2 h after the addition of the inducers.

tested, neither paraquat nor H_2O_2 was capable of inducing the expression of the *yqfS-lacZ* fusion.

Several *B. subtilis* genes involved in DNA repair, such as *uvr* components and *recA*, have been shown to be inducible not only by DNA damage but also by the physiological state of competence (32, 34, 48). These genes (*din*) are part of a global response which in *B. subtilis* is called the SOS response (33). In order to determine whether the type IV AP-endonuclease gene of *B. subtilis* is a component of the *B. subtilis* SOS regulon, the strain containing the *yqfS-lacZ* fusion was grown to exponential phase and then treated with mitomycin C to a final concentration of 0.5 μg/ml. As shown in Fig. 9B, mitomycin C induced the β-galactosidase levels of the strain *B. subtilis* PERM317 only 1.2 times above the levels expressed by the untreated control. In contrast with this result, when *B. subtilis* YB3000 containing a *recA-lacZ* fusion was treated with mitomycin (Fig. 9C), the levels of β-galactosidase activity increased 35 times.

DISCUSSION

B. subtilis has been studied extensively as a paradigm for bacterial differentiation and development. Spores produced by this organism prevent or dramatically slow the DNA damage inflicted by oxidative stress, UV light, heat and desiccation (reviewed in references 40 and 58). However, during long periods of dormancy spores accumulate potentially lethal and mutagenic DNA damage such as SP, strand brakes, CPDs, chemically altered bases, and AP sites that could affect transcription and replication during germination (40, 56). Therefore, it is of interest to determine how the many DNA repair systems present are regulated by *B. subtilis*, especially in relation to the sporulation and germination processes.

Thus, the *yqfS* ORF was cloned, and the product of this gene was isolated and tested for its enzymatic activity. The results presented in Fig. 2 and 3 clearly indicate that this protein has AP endonuclease activity. Having established the nature of the product of the *yqfS* gene, we wanted to determine the mechanism(s) that control the expression of this gene. Our data demonstrate that there is temporal and spatial expression of the *yqfS* gene. Specifically, β-galactosidase activity for a *yqfS-LacZ* reveals that this gene is not apparently transcribed during vegetative growth but is transcribed during stages of the sporulation process (Fig. 4). Northern blot and RT-PCR experiments (Fig. 5) confirmed a major abundance of *yqfS* messengers during stages of the sporulation process of the strain *B. subtilis* PERM317. These results suggested that *yqfS* expression is temporally activated and confined to the forespore compartment in accordance with a pattern similar to that described for stage III, forespore-specific genes (57). This suggestion was further supported not only by cell fractionation experiments, which demonstrated that *yqfS* expression occurs inside of the spore, but also by the observation that the kinetics of GDH synthesis, a stage III, forespore-specific marker, are indistinguishable from those observed for the *yqfS-lacZ* fusion (Fig. 4). These results strongly support the idea that the synthesis of the YqfS protein occurs during the last stages of the sporulation process and is packaged in the spore.

The forespore-specific expression of the *yqfS-lacZ* fusion during the last steps of *B. subtilis* sporulation suggested that the transcription of *yqfS* is carried out by RNA polymerase containing the σ^G factor (Fig. 4). However, gene expression inside of the forespore occurs by the sequential action of two RNA polymerases containing either the σ^F or σ^G factors (21, 25). Therefore, we could not rule out a possible transcription of *yqfS* by RNA polymerase σ^F. This point was addressed by measuring the levels of expression of the *yqfS-lacZ* fusion introduced into a *B. subtilis* strain lacking the *sigG* gene (Table 1). The results showed that *yqfS*-directed β-galactosidase activity is almost null in this genetic background, as is the synthesis of GDH activity (data not shown). In agreement with this observation, both Northern blot and RT-PCR experiments performed with total RNA isolated during vegetative and stationary growth of the strain *B. subtilis sigGΔ1* demonstrated the absence of *yqfS* messengers in this mutant strain (Fig. 6). Taken collectively, these results strongly suggest that *yqfS* expression occurs inside of the spore by the action of σ^G-containing RNA polymerase.

Forespore-specific expressed genes such as *sspA-E*, *splA-splB*, *gdh*, *ger*, and *spoVA*, among others, are representative of the σ^G regulon (15, 16, 19, 21, 25, 44, 45, 57). Experimental evidence has demonstrated that these genes possess specific promoters that are exclusively transcribed by σ^G containing RNA polymerase (15, 39, 43, 44, 47). The results described above suggest that *yqfS* might be a new member of this regulon. This conclusion was strongly supported by the in vivo mapping of the transcriptional start site of *yqfS* (Fig. 7). A major extension product initiating 54 to 55 bp upstream of the putative *yqfS* start codon was amplified from RNA samples isolated from sporulating but not from vegetatively growing cells (Fig. 7). Inspection of the sequences preceding the *yqfS* transcriptional start site revealed the existence of a promoter with homology to the consensus sequence of σ^G promoters (21, 43). Although the −10 region of the putative *yqfS* promoter shows a low level of homology, the −35 region almost perfectly matched the consensus of σ^G promoters (Fig. 8). One possible problem with the designation of this putative σ^G promoter is the spacing between the −35 and −10 regions. However, as mentioned above, our data support the hypothesis that the *yqfS* gene is transcribed by a σ^G-containing RNA polymerase.

The *yqfS* region in the *B. subtilis* chromosome shows the existence of a set of three genes located in the same orientation, in the following order: *yqfR*, *yqfS*, and *yqfU* (Fig. 1). The lack of putative transcriptional terminators downstream of *yqfR* and *yqfS* suggests that the three genes are transcribed as a polycistronic unit. However, the primer extension experiments described above, together with the identification of a 2.3-kb *yqfS* messenger, indicate that *yqfS* is cotranscribed with *yqfU* as a bicistronic mRNA from the putative *yqfS* promoter just described.

Expression of the two major AP endonucleases is differentially regulated in *E. coli*. Whereas *exoIII* is constitutively expressed, the *nfo* gene is inducible by oxidative stress. Chemical compounds such as paraquat and menadione, which generate superoxide radicals, induce a 10- to 20-fold increase in the level of Nfo (8). The lack of induction in the levels of expression of the *yqfS-lacZ* fusion after the treatment of *B. subtilis* PERM317 with paraquat (Fig. 9) revealed that in *B. subtilis* the *yqfS* gene is not regulated by the oxidative stress imposed by superoxide radicals.

In *B. subtilis* the adaptive response to H_2O_2 stress is subjected to negative regulation by the repressor PerR, a Fur homolog (6). Treatment of *B. subtilis* PERM317 with H_2O_2 did not change the levels of expression of the *yqfS-lacZ* fusion (Fig. 9), suggesting that *yqfS* is not regulated by PerR. Consistent with these results, no *cis*-acting DNA sequences similar to those present in *perR* boxes (22) were observed around the putative promoter of *yqfS*.

Analysis of the upstream regions of *yqfS* also revealed the absence of *dinR*-like boxes (11, 62). This observation is in agreement with the lack of induction of the *yqfS-lacZ* fusion after the treatment of *B. subtilis* PERM317 with the DNA-damaging agent mitomycin (Fig. 9).

Taking all of these results together, we conclude that although in *E. coli* the expression of *nfo* is linked to the oxidative stress generated by superoxide radicals (8), in *B. subtilis* the regulation of *yqfS* expression occurs in a temporal and forespore-specific manner and appears to be part of the σ^G regulon. In addition, the lack of induction of β-galactosidase in the *yqfS-lacZ* fusion strains after treatment by either hydrogen peroxide or the DNA-damaging agent mitomycin revealed that *yqfS* in not under the control of the PerR or SOS regulons.

Despite the existence of spore mechanisms that prevent or alter DNA insults, potentially lethal and mutagenic damage accumulates in DNA during long-term storage of spores in the laboratory (40, 58) and during the exposure of these spores to environmental stresses, particularly solar radiation (40, 41, 59, 61). Interestingly, artificial and solar UV radiation induce the formation of SP, CPDs, and strand breaks but not of AP sites in *B. subtilis* spore DNA (59). It remains to be investigated whether AP sites are generated during germination of *B. subtilis* spores either spontaneously or promoted by oxidative stress or through the action of DNA glycosylases during the elimination of chemically modified bases (18). Moreover, depending on their chemical structure, single-strand breaks generated on spore DNA could be processed as well by YqfS during germination, since it has been well established that type IV AP endonucleases are able to remove phosphoglycoaldehyde, phosphate, deoxyribose-5-phosphate, and 4-hydroxy-2-pentenal from the 3′ terminus of duplex DNA (18). Therefore, as an obligatory step for the correction of the different types of DNA damage processed by the BER pathway, YqfS may play an important role in the repair of DNA damage inflicted on *B. subtilis* during either spore dormancy or germination.

In conclusion, we provide here for the first time evidence that an important component of the BER system of *B. subtilis*, namely, the *yqfS* gene, is specifically expressed inside of the spores during the final developmental stages. Thus, together with the SplB, UVR, and Rec systems, YqfS could be part of the DNA repair proteins that increase the survival potential of *B. subtilis* spores.

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