The *ytkD* (*mutTA*) Gene of *Bacillus subtilis* Encodes a Functional Antimutator 8-Oxo-(dGTP/GTP)ase and Is under Dual Control of Sigma A and Sigma F RNA Polymerases

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The regulation of expression of *ytkD*, a gene that encodes the first functional antimutator 8-oxo-dGTPase activity of *B. subtilis*, was studied here. A *ytkD-lacZ* fusion integrated into the *ytkD* locus of wild-type *B. subtilis* 168 revealed that this gene is expressed during both vegetative growth and early stages of sporulation. In agreement with this result, *ytkD* mRNAs were detected by both Northern blotting and reverse transcription-PCR during both developmental stages. These results suggested that *ytkD* is transcribed by the sequential action of RNA polymerases containing the sigma factors σ^A and σ^F , respectively. In agreement with this suggestion, the spore-associated expression was almost completely abolished in a *sigF* genetic background but not in a *B. subtilis* strain lacking a functional *sigG* gene. Primer extension analysis mapped transcriptional start sites on mRNA samples isolated from vegetative and early sporulating cells of *B. subtilis*. Inspection of the sequences lying upstream of the transcription start sites revealed the existence of typical σ^A - and σ^F -type promoters. These results support the conclusion that *ytkD* expression is subjected to dual regulation and suggest that the antimutator activity of YtkD is required not only during vegetative growth but also during the early sporulation stages and/or germination of *B. subtilis*. While *ytkD* expression obeyed a dual pattern of temporal expression, specific stress induction of the transcription of this gene does not appear to occur, since neither oxidative damage (following either treatment with paraquat or hydrogen peroxide) nor mitomycin C treatment or σ^B general stress inducers (sodium chloride, ethanol, or heat) affected the levels of the gene product produced.

Reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide, and the hydroxyl radical, generated as by-products of cellular metabolism (56, 57), have the potential to react with proteins, lipids, and DNA (48, 50, 55). Oxidative damage to DNA can result in the generation of apurinic/apirimidinic (AP) sites, several types of base modification, sugar damage and single- and double-strand breaks (22). Furthermore, the intracellular deoxyribonucleotide and ribonucleotide pools are also potential targets of oxidative damage (30, 59). Thus, as a consequence of ROS action, dGTP and GTP can be converted to 8-oxo-dGTP and 8-oxo-GTP, respectively (59). The former can be incorporated into nascent DNA strands opposite adenine, thus playing a significant role in mutagenesis, aging, and cancer (38). On the other hand, it has been proposed that 8-oxo-GTP has the potential of being incorporated into mRNAs, generating transcriptional errors (59). To counteract the potential mutagenic effects of 8-oxo-dGTP and transcriptional errors induced by 8-oxo-GTP, cells rely on the protein MutT, which degrades the oxidized nucleotides to the corresponding monophosphate forms. These chemical changes prevent the incorporation of the altered nu-

cleotides into either DNA or mRNA, respectively (59). Proteins with 8-oxo-dGTPase activity have been described and isolated from bacteria (6, 11, 29) as well as from mammalian cells (28, 42, 58).

Multiple mechanisms act together to prevent or repair oxidative damage to DNA in the gram-positive spore-forming bacterium *Bacillus subtilis*, and the genes that encode such mechanisms have shown to be temporally regulated during vegetative growth and postexponential differentiation. For instance, *yqfS*, which codes for a type IV AP endonuclease and is a component of the base excision repair pathway, is expressed in the forespore compartment of the sporulating bacteria under the control of σ^G RNA polymerase [E(sig)G] (60). On the other hand, *katA*, *katB*, and *katX* encode catalases of *B. subtilis*, and these genes, while also subjected to differential regulation, are not always transcribed in response to various developmental stages. For instance, *katA* is expressed during vegetative growth and following bacterial treatment with H_2O_2 (7), while the expression of *katB* and *katX* is controlled by the stress-regulated σ^B (19) and the spore-specific σ^F (4), respectively. An extremely important reactive component of cells is the superoxide radical. *B. subtilis* seems to possess a single superoxide dismutase (SOD) gene called *sodA* (12, 27), and this gene is expressed in all phases of growth and during sporulation from different promoters (12, 27).

As mentioned above, the product 8-oxo-dGTP is known to

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Source
Strains		
<i>B. subtilis</i>		
168	<i>trpC2</i>	W. L. Nicholson
1S86	<i>sigF1 trpC2</i>	BGSC ^a
WN118	<i>sigGΔ1 trpC2</i>	W. L. Nicholson
PERM276	<i>trpC2 ytkD-lacZ</i> Cm ^r	This study
PERM346	<i>sigF1 trpC2 ytkD-lacZ</i> Cm ^r Kan ^r	This study
PERM 333	<i>sigGΔ1 trpC2 ytkD-lacZ</i> Cm ^r	This study
YB3000	<i>metB5 trpC2 xin-1 sigB amyE</i> (deleted for spβ) pCCR202	R. E. Yasbin
<i>E. coli</i>		
SURE	e14 ⁻ (McrA ⁻) Δ(<i>mcrCB-hsdSMR-mrr</i>)171 <i>endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ</i> <i>sbhC umuC::Tn5</i> (Kan ^r) <i>uvrC</i> [F' <i>proAB lacI^qlacZΔM15 Tn10</i> (Tet ^r)]	Stratagene
PERM162	<i>E. coli</i> SURE, pUC18, Amp ^r Tc ^r	This study
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacI^qΔ M15 Tn10</i> (Tet ^r)	Stratagene
PERM254	<i>E. coli</i> XL1-Blue, pPERM254, Amp ^r	This study
PERM274	<i>E. coli</i> XL1-Blue, pPERM274, Amp ^r	This study
XL10-Gold Kan	Tet ^r Δ(<i>mcrA</i>) 183 Δ(<i>mcrBC-hsd SMR-mrr</i>) 173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lacHte</i> [F' <i>proAB lacI^qZDM15 Tn10</i> (Tet ^r) Tn5 (Kan ^r) Amy]	Stratagene
PERM375	<i>E. coli</i> XL10-Gold, pPERM375, Amp ^r <i>guaC purM mutT1</i>	This study
SB3		M. J. Bessman
JM83	F ⁻ <i>ara</i> Δ(<i>lac-proAB</i>) <i>rpsL</i> (Str ^r) [φ80 <i>dlac</i> Δ(<i>lacZ</i>)M15] <i>thi</i>	Laboratory stock
PERM279	SURE, pPERM279, Amp ^r	This study
PERM280	SURE, pPERM280, Amp ^r	This study
PERM425	SB3, pTrc99A, Amp ^r	This study
PERM426	SB3, pPERM426, Amp ^r	This study
PERM427	XL10-Gold, pPERM427, Amp ^r Kan ^r Tet ^r	This study
Plasmids		
pJF751	Integral <i>lacZ</i> fusion vector, Cm ^r	W. L. Nicholson
pUC18	Multisite <i>E. coli</i> cloning vector	Laboratory stock
pPERM254	<i>ytkD</i> gene cloned in pUC18	This study
pPERM274	338-bp <i>EcoRI-AviI</i> fragment of <i>ytkD</i> from pPERM254 cloned in pJF751	This study
pTrc99A	IPTG-inducible promoter vector for expression, Amp ^r	Laboratory stock
pOE30	IPTG-inducible promoter vector for expression, Amp ^r	Laboratory stock
pPERM279	PCR-amplified <i>ytkD</i> cloned in <i>Bam</i> HI site of pUC18	This study
pPERM280	PCR-amplified <i>ytkD</i> cloned in <i>Xba</i> I- <i>Bam</i> HI site of pUC18	This study
pPERM426	<i>ytkD</i> from pPERM280 gene cloned in <i>Xba</i> I- <i>Bam</i> HI site of pTrc99A	This study
pPERM427	<i>ytkD</i> from pPERM280 gene cloned in <i>Bam</i> HI site of pTrc99A	This study

^a BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

be problematic with respect to mutagenesis and survival in living cells. Therefore, it is not surprising that the genome of *B. subtilis* (33) possesses the genes *yqkG* (*nudF*), *mutT*, *yvcI*, *yjhV*, and *ytkD*, whose reading frames encode potential homologs of the *Escherichia coli* MutT protein, the archetype of the 8-oxo-dGTPases (1). A recent study revealed that *nudF* encodes a nucleotide diphosphohydrolase (Nudix) with specificity to split ADP-ribose into AMP and ribose-5-phosphate (18). Thus, the product of the *nudF* gene is not believed to be associated with conferring protection to *B. subtilis* against the mutagenic effects of the oxidized nucleotide 8-oxo-dGTP. Accordingly, the identification and characterization of the proteins involved in protecting *B. subtilis* from the mutagenic effects of oxidized nucleotide pools generated by ROS action remain to be established.

YtkD possesses a 23-amino-acid-long sequence that contains 9 of the 10 absolutely conserved residues in the Nudix amino acid signature of all proteins that have been shown to hydrolyze 8-oxo-dGTP (5, 23). In this communication, we report that *ytkD* not only encodes the first reported 8-oxo-dGTPase of *B. subtilis* but also possessed the ability to com-

plement the mutator phenotype of an *E. coli* *mutT* mutant. Further evidence provided here demonstrated that while the transcription of *ytkD* followed a dual pattern of temporal expression controlled by the sequential action of σ^A - and σ^F -containing RNA polymerases, the transcription of this gene was not stimulated by oxidative damage or by inducers of the SOS or σ^B general stress responses

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *B. subtilis* and *E. coli* strains and the plasmids used in this study are shown in Table 1. Difco sporulation medium (DSM) (52) and Luria-Bertani medium (LB) (41) were used to propagate *B. subtilis* and *E. coli* strains, respectively. When required, antibiotics were added to media at the following final concentrations: chloramphenicol, 3 μ g/ml; ampicillin, 50 μ g/ml; and kanamycin, 10 μ g/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 were performed as previously described (8, 49). Chromosomal DNA from *B. subtilis* was purified according to the protocol of Cutting and Vander Horn (16). Small-scale preparation of plasmid DNA from *E. coli* cells, enzymatic manipulations, and agarose gel electro-

phoresis were performed by standard techniques (49). Medium-scale preparation and purification of plasmid DNA were 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 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.

Complementation of an *E. coli* *mutT* mutant by *ytkD* expression. *E. coli* strain SB3 (kindly provided by M. J. Bessman, The John Hopkins University), lacking a functional MutT protein, was transformed with either pTrc99A or pPERM426 (pTrc99A-*ytkD*) (Table 1). The resulting strains PERM425 and PERM426 were grown for 24 h in LB medium containing 100- μ g/ml ampicillin and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Additionally, strains *E. coli* SB3 and *E. coli* JM83 were grown in LB medium in the absence of antibiotics. Mutation frequencies were determined by plating aliquots on LB plates containing nalidixic acid at 20 μ g/ml. The mutant colonies were counted after 1 day of incubation at 37°C to estimate mutation frequencies.

Design of a plasmid to overexpress *ytkD* and purify a His₆-YtkD protein. The open reading frame (ORF) of *ytkD* lacking the first codon and extending 90 bp downstream of the stop codon was amplified by PCR utilizing Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and oligonucleotide primers that inserted *Bam*HI restriction sites into the cloned DNA. The PCR DNA fragment was first ligated into *Hinc*II-treated pUC18 and replicated into *E. coli* SURE (Stratagene, La Jolla, Calif.). The resulting construct (pPERM279) was cut with *Bam*HI, and the 564-bp *ytkD* insert was cloned in-frame into *Bam*HI-treated pQE30 to generate pPERM427, which was transformed into *E. coli* XL-10 Gold Kan (Stratagene).

Purification of His₆-YtkD. *E. coli* PERM427 was grown at 37°C in 50 ml of LB medium supplemented with ampicillin to an OD of 0.5. Expression of the *ytkD* gene was induced during 4 h of incubation at 28°C by the addition of IPTG (0.5 mM). Cells were collected by centrifugation and washed two times with 10 ml of 50 mM Tris-HCl (pH 7.5) mixed with 300 mM NaCl (buffer A). The cells were frozen at -70°C for 12 h and disrupted by defrosting at room temperature. The cell homogenate was resuspended in 10 ml of buffer A containing 1% (vol/vol) Triton X-100, incubated for 30 min on ice, and then subjected to centrifugation (27,200 $\times g$ for 10 min) to eliminate undisrupted cells and cell debris, and the supernatant was applied to a 3-ml Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen) column, previously equilibrated with buffer A. The column was washed with 100 ml of buffer A containing 20 mM imidazole plus 100 ml of buffer A containing 30 mM imidazole, and the protein bound to the resin was eluted with 10 ml of buffer A containing 100 mM imidazole; 2-ml fractions were collected during this step.

Assay of enzymatic activity. A colorimetric assay (described below) was used to measure the relative rates of hydrolysis of dGTP, GTP (purchased from Roche, Mannheim, Germany), 8-oxo-dGTP, and 8-oxo-GTP (purchased from JENA Bioscience, Jena, Germany). The nucleoside triphosphatase (NTP) activity of YtkD was measured in a 50- μ l reaction mixture containing the following components: 40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 4 mM the appropriate NTP, 10 mM dithiothreitol (DTT), and 0.5 U of yeast inorganic pyrophosphatase. A unit of pyrophosphatase hydrolyzes 1 μ mol of PP_i to P_i per min at 25°C. The reaction was allowed to proceed for 15 min at 37°C before being terminated by the addition of 50 μ l of a 4:1 mixture of a 20% suspension of Norit A and 7% perchloric acid. This was mixed and allowed to stand for 5 min on ice before centrifugation to sediment the Norit A and absorbed nucleotides. An aliquot of the supernatant was then used to determine the amount of free inorganic orthophosphate by the method of Ames and Dubin (2). A unit of enzyme activity hydrolyzes 1 μ mol of substrate per min.

Construction of a *B. subtilis* strain containing a *ytkD-lacZ* gene fusion. Construction of an in-frame translational fusion between the *ytkD* gene and the *E. coli* *lacZ* gene was carried out in the integrative plasmid pJF751 (20) by inserting a 338-bp *Eco*RI-*Avi*I fragment from plasmid pPERM254 into pJF751 previously digested with *Eco*RI and *Sma*I. The resulting construct, containing the *ytkD-lacZ* fusion and designated pPERM274, was propagated into *E. coli* XL1-Blue. Plasmid pPERM274 was introduced by transformation into competent cells of *B. subtilis* strains 168, 1S86 (*sigF* mutant), and WN118 (*sigG* mutant), and transformants were selected on solid DSM containing chloramphenicol.

Cell growth and enzymatic assays. *B. subtilis* strains carrying the *ytkD-lacZ* fusion were grown and allowed to sporulate in liquid DSM containing chloramphenicol. Samples of 1.5 ml were collected during vegetative growth and throughout sporulation. Cells were washed with cold 0.1 M Tris-HCl (pH 7.5), and the cell pellets were stored at -20°C until determination of β -galactosidase activity (43, 46). Briefly, washed cell samples were first disrupted with lysozyme

and subjected to centrifugation. β -Galactosidase activity present in the supernatant was measured and assigned to the mother cell fraction (which actually consisted of mother cells plus lysozyme-sensitive forespores). The pellet, which consisted of lysozyme-resistant forespores containing spore coats, was subjected to spore coat removal (43) and washed in 50 mM Tris-HCl (pH 7.5) buffer, and a second round of lysozyme treatment was assigned to the forespore fraction for determination of β -galactosidase activities (39, 43).

Induction experiments. Experiments were performed to analyze whether paraquat, H₂O₂, ethanol, NaCl, mitomycin C, or heat (48°C) induced the expression of the *ytkD-lacZ* fusion of the strain *B. subtilis* PERM276. Each compound was tested independently as follows. Cells were grown in LB medium (lacking NaCl) to an OD at 600 nm (OD₆₀₀) of 0.5. The culture was divided into two subcultures of equal volume, and each of the compounds described above was added to one subculture to the following final concentrations: paraquat, 10 μ M; H₂O₂, 200 μ M; NaCl, 4%; ethanol, 4%; and mitomycin C, 0.5 μ g/ml. The second subculture served as a control. Induction by heat was carried out by incubating the experimental culture, with aeration, at 48°C. Cells were harvested after 1 h of induction and assayed for β -galactosidase activity.

Northern blot and primer extension experiments. The total RNA from vegetative and sporulating cells of strains *B. subtilis* 168 and 1S86 was isolated as previously described (39). RNA samples (40 μ g) were separated by electrophoresis through a 1% agarose-formamide gel and transferred to a high-bound nylon membrane. The membrane containing the transferred RNA was hybridized at 60°C with a 680-bp *Eco*RI-*Bam*HI fragment from pPERM279 encompassing the entire *ytkD* sequence. The probe was labeled by random priming with [α -³²P]dCTP by using the Rediprime II DNA labeling system according to the instructions of the provider (Amersham Bioscience, Buckinghamshire, England). Detection of hybrids was performed by autoradiography following exposure of the membranes to Kodak X-Omat films. The size of the hybrids was estimated by using RNA markers (Promega, Madison, Wis.) of 281, 623, 955, 1,383, 1,517, 1,908, 2,604, 3,638, and 4,981 nucleotides (nt), respectively.

The 5' ends of *ytkD* were mapped by primer extension (40) of *ytkD* transcripts produced during both vegetative growth and sporulation. To this end, total RNA was isolated (39) from vegetative and sporulating cells (stage T₅ [5 h after the end of log-phase growth]) of *B. subtilis* 168. The total RNA (40 μ g from each sample) was hybridized with the 23-mer oligonucleotide 5'-CCAGACATGCTTCGGGC TGTCCG-3', which was complementary to the *ytkD* mRNA from nt 66 to 88 downstream from the putative *ytkD* translational start codon. The oligonucleotide was labeled on its 5' end with [γ -³²P]ATP and T4 polynucleotide kinase. The primer was extended with Moloney murine leukemia virus reverse transcriptase (Promega), 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 23-base primer and as template DNA a 1,409-bp PCR product (PCR) extending from 866 bp upstream and 543 bp downstream of the start codon of *ytkD*.

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 the Master AMP RT-PCR kit (Epicentre Technologies) according to the instructions of the provider. The primers used for RT-PCRs were 5'-GCTCTAGAGGGATAAACATGTACGAG-3' (forward) and 5'-CTTCTGCGCACTCCATCGGCTCTAG-3' (reverse) to generate a 204-bp RT-PCR product extending from 17 bp upstream from the start codon of *ytkD* to 187 bp downstream of this point. As a control, in each experiment, the absence of chromosomal DNA in the RNA samples was assessed by PCRs carried out with Vent DNA polymerase (New England Biolabs) and the set of primers described above. The size of the RT-PCR product was assessed by utilizing the 1-kbp-plus DNA ladder (Life Technologies, Rockville, Md.).

RESULTS

Enzyme activity of YtkD. The antimutator effects of MutT homologs rely on the ability of these proteins to catalyze the conversion of 8-oxo-dGTP into its monophosphate form (38). As shown in Fig. 1B, the five potential MutT homologs from *B. subtilis* conserve a 23-amino-acid-long signature termed the MutT or Nudix box (5, 23), which keeps a high level of identity (47.8 to 56.5%) with the MutT box of the *E. coli* MutT protein. However, not all of the proteins that conserve such a signature

FIG. 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of His₆-YtkD purification through an Ni-NTA-agarose column. Fifteen-microliter aliquots of each sample were electrophoresed on a 12% polyacrylamide gel, which was stained with Coomassie blue. Lanes: 1, molecular weight standards; 2, noninduced *E. coli* PERM427 lysate; 3, IPTG-induced *E. coli* PERM427 lysate; 5 to 9, fractions eluted from the column with 100 mM imidazole. (B) Comparison of the 23-amino-acid-long MutT boxes of YtkD, MutT, YvcI, YjhV, and NudF from *B. subtilis* and MutT homologs with a proven 8-oxo-dGTPase activity (23). Alignment was performed with MegAlign (Clustal method) of the DNASTAR program. Asterisks mark residues absolutely conserved in all of the MutT homologs that hydrolyze 8-oxo-dGTP (23).

have been shown to be capable of hydrolyzing 8-oxo-dGTP or complementing the mutator phenotype of a *mutT*-deficient *E. coli* strain (21, 45). Therefore, we first investigated whether YtkD catalyzed the hydrolysis of 8-oxo-dGTP. To this end, the ORF of *ytkD* was expressed from the IPTG-inducible T₅ promoter of plasmid pQE30 in order to generate a recombinant protein containing an N-terminal His₆ tag. The His₆-YtkD protein was successfully synthesized in *E. coli* cells and purified to apparent homogeneity by metal chelate affinity on an Ni-NTA-agarose column (Fig. 1A). As shown in Table 2, the purified His₆-YtkD protein successfully catalyzed the degradation of 8-oxo-dGTP. The results indicated that YtkD catalyzed the hydrolysis of 8-oxo-dGTP with a specific activity 413 times higher than that exhibited against dGTP. It has been demonstrated that MutT from *E. coli* also possesses enzymatic activity against 8-oxo-GTP (59). Therefore, we investigated whether

YtkD utilized this oxidized mRNA precursor as a substrate. The results shown in Table 2 demonstrated that 8-oxo-GTP is indeed a much better substrate for YtkD hydrolysis than GTP. The specific activity of YtkD during degradation of oxidized GTP was around 460 times higher than that obtained against

TABLE 2. Substrate specificity of His ₆ -YtkD		
Substrate ^a	Sp act (U mg ⁻¹) ^b	Relative activity (%)
8-Oxo-dGTP	37.2 ± 1.042	100
dGTP	0.09 ± 0.016	0.24
8-Oxo-GTP	36.8 ± 0.142	98.8
GTP	0.08 ± 0.026	0.21

^a Nucleotides were present at a concentration of 1 mM, and 17.7 ng of enzyme was used in each reaction.

^b One unit of enzyme hydrolyzes 1 μmol of substrate per min.

GTP (Table 2). These results demonstrated that YtkD is a protein with the enzyme properties required to preferentially catalyze the hydrolysis of 8-oxo-dGTP and 8-oxo-GTP. Such catalytic activity strongly suggests that this protein's potential physiological role is to counteract the mutagenic effects of these oxidized nucleotides. In agreement with these ideas, a $\Delta ytkD$ *B. subtilis* strain that was recently constructed in our laboratory was demonstrated to be 1 order of magnitude more mutagenic than its parental strain (F. X. Castellanos-Juárez and M. Pedraza-Reyes, unpublished results).

Genetic complementation of the *E. coli* *mutT* mutation by *ytkD*. To further support the contention that *ytkD* encodes a physiologically functional 8-oxo-dGTPase, we investigated whether the expression of *ytkD* complemented the mutator phenotype of *E. coli* cells lacking a functional *mutT* gene. It has been demonstrated that the occurrence of A:T-to-G:C transversion is several orders of magnitude greater in *E. coli* strains deficient in MutT activity than the numbers seen in isogenic wild-type bacteria (22, 38). Accordingly, the ORF of *ytkD* was cloned in pTRC99A, and the resulting plasmid, pPERM426, was introduced by transformation into the *mutT* mutant strain in order to examine the effects of *ytkD* expression on the levels of the spontaneous mutation frequency for nalidixic acid resistance. As shown below, the mutation frequency of the bacteria lacking a functional *mutT* gene and expressing *ytkD* was significantly lower than that of the *mutT* mutant bacteria carrying only the pTRC99A vector. For *E. coli* strains JM83 (wild type), SB3 (*mutT* mutant), PERM425 (SB3 + pTRC99A), and PERM426 (SB3 + pTRC99A-*ytkD*), the mutation frequencies (Nal^r) per 10^8 cells were 0.01 ± 0.009 , 8.30 ± 1.21 , 7.10 ± 1.33 , and 0.819 ± 0.22 , respectively. (Mean values of mutation frequencies were calculated from at least four independent experiments.) A similar result was previously obtained when the cDNA encoding the MutT human homolog was used in this complementation experiment (58). Taken together, these results demonstrate that YtkD not only degrades 8-oxo-dGTP and 8-oxo-GTP but also genetically complements the mutagenic effects of the *mutT* deficiency in *E. coli*.

Expression of *ytkD* during growth and sporulation. To analyze the pattern of expression of *ytkD*, the *B. subtilis* strain PERM276 harboring a single copy of the *ytkD-lacZ* fusion was induced to sporulate in DSM. The *ytkD*-directed β -galactosidase activity was detected during both vegetative growth and sporulation (Fig. 2). In the nonspore fraction, the activity was found to be present during growth and then began to increase as the cells entered stationary phase, followed by a marked decrease between stages T_4 and T_5 (Fig. 2). This pattern of expression suggested a compartmental expression of the *ytkD* gene into the forespore. As shown in Fig. 2, β -galactosidase activity was indeed detected in the forespore fraction from sporulation stages T_4 to T_5 and continued to accumulate until at least stage T_8 .

The dual pattern of temporal expression of *ytkD* was further corroborated as follows. First, Northern blot experiments were performed with total RNA isolated from cells of the strain *B. subtilis* 168 collected before and after the onset of sporulation. The results shown in Fig. 3A indicated that *ytkD* mRNA appeared as a major ~ 0.5 -kb band during both vegetative growth and sporulation (T_0 to T_3). A minor hybridization band was also observed in this experiment, which could correspond to a

FIG. 2. Expression of a *ytkD-lacZ* translational fusion during growth and sporulation of *B. subtilis*. *B. subtilis* PERM276 was grown to sporulation in liquid DSM (●). Samples were collected at different times and treated with lysozyme, and the extracts were assayed for β -galactosidase (▲). The β -galactosidase activity inside of the forespore lysozyme-resistant fraction (◆) was assayed as described in Materials and Methods. The results are representative, and the experiments were performed at least three times.

degradation product from the major band, because the former was not observed in the Northern blot experiments performed with mRNAs isolated from a *sigF* *B. subtilis* strain (Fig. 3C).

In addition, following RT-PCR experiments with total RNA samples isolated from both vegetative and sporulating cells (Fig. 3B), the resulting amplification products both had the expected molecular size of 204 bp. However, the 204-bp RT-PCR product was more abundant when RNA samples from growth stages T_3 to T_5 of sporulation were utilized in the assays. Taken together, these results are consistent with the existence of a double level of regulation of *ytkD* transcription: one associated with vegetative growth and a second putative forespore mechanism that controls the expression of *ytkD* during sporulation.

Sigma factor dependence of *ytkD* expression. The transcription of genes in the forespore compartment of *B. subtilis* is carried out through the sequential action of two temporally expressed RNA polymerases containing either the σ^F or σ^G factors (24, 31, 32). However as shown in Fig. 2, the pattern of expression of the *ytkD*-directed β -galactosidase activity during sporulation suggested that the transcription of *ytkD* might be under control of the E(sig)F form of the RNA polymerase. To better investigate this notion, a single copy of the *ytkD-lacZ* fusion was introduced, by transformation, into strains *B. subtilis* 1S86 and WN118, which lack a functional SigF or SigG, respectively. Our results demonstrated that a mutation in the *spoIIAC* gene, which encodes the forespore-specific σ^F (34, 51), drastically reduced the expression of the *ytkD-lacZ* fusion during sporulation but not during vegetative growth (Fig. 4A). On the other hand, the expression of the *ytkD*-directed β -galactosidase activity during either vegetative growth or sporulation (Fig. 4B) was not affected in the strain that lacked a functional SigG activity. Furthermore, when Northern blot ex-

FIG. 3. Northern blot (A and C) and RT-PCR analysis (B) of *ytkD* transcription during vegetative (Veg) growth and sporulation of *B. subtilis* 168 (wild type; A and B) and *B. subtilis* IS68 (*sigF*, C). (A and C) *B. subtilis* 168 and IS68 were grown in liquid DSM. Total RNA was isolated during the steps indicated. Samples of around 40 μ g were separated on agarose-formaldehyde gels (lower panel, 16S and 23S rRNA bands) and transferred to nylon membranes. The membrane was hybridized with a 32 P-labeled, 1,181-bp fragment encompassing the entire *ytkD* sequence as described in Materials and Methods. (B) RNA samples (1 μ g) isolated from a *B. subtilis* 168 DSM culture, at the steps indicated, were processed for RT-PCR analysis as described in Materials and Methods. The arrowhead shows the size of the expected RT-PCR product.

periments were performed with RNA isolated from vegetative and stationary-phase cells of the SigF-deficient strain grown in liquid DSM, only a strong hybridization signal of the expected size was observed with RNA samples isolated from vegetatively growing cells (Fig. 4C, lane 1) and not with those isolated from the stationary phase of growth (Fig. 4C, lanes 2 to 6). Therefore, we conclude that the transcription of the *ytkD* gene associated with sporulation is dependent on the E(sig)F form of the RNA polymerase.

Mapping the transcriptional start sites of *ytkD*. The reported genome of *B. subtilis* (<http://genolist.pasteur.fr/SubtiList/>) demonstrates that the *ytkD* gene is flanked upstream by *ytkC*, which encodes a putative protein of unknown function, similar to an autolytic amidase. Despite the existence of a 208-bp-long intergenic region between *ytkC* and *ytkD*, no potential transcriptional terminators are reported (<http://genolist.pasteur.fr/SubtiList/>) until the end of *ytkD*. This gene arrangement suggests that *ytkC* and *ytkD* might form part of a bicistronic

operon. To investigate whether *ytkC* and *ytkD* are coexpressed as part of the same mRNA, the 5' ends of *ytkD* were mapped in vivo by primer extension (40). Accordingly, primer extension analysis was performed with total RNA samples isolated from *B. subtilis* 168 cells during vegetative growth, at the point at which the culture ceased exponential growth (T_0) and 5 h later (T_5). As shown in Fig. 6B, when total RNA isolated from vegetative cells was used as a template, a major extension product was detected, located 31 to 32 bp upstream of the *ytkD* start codon (Fig. 5B; lane 3). A smaller amount of this product was also detected in RNA samples of stage T_0 (Fig. 5B, lane 2). Analysis of the regions lying upstream of this *ytkD* transcription start site revealed the existence of sequences with good homology to promoters of σ^A -dependent genes (Fig. 6A).

Interestingly, as shown in Fig. 5A, a second major extension product of *ytkD* was obtained with RNA samples from T_5 (Fig.

FIG. 4. Expression of a *ytkD-lacZ* translational fusion in *B. subtilis* *sigF* and *sigG* genetic backgrounds. *B. subtilis* strains PERM346 (A; *sigF*) and PERM333 (B; *sigG*) were grown in liquid DSM (●). Samples were collected at different times and treated with lysozyme, and the extracts were assayed for β -galactosidase (▲). The results are representative, and the experiments were performed at least three times.

FIG. 5. Primer extension analysis for mapping the transcriptional start site of *yt**kD*. Total RNA was isolated from either vegetative (lane 3), stage *T*₀ (lane 2), or sporulating (stage *T*₅; lane 1) *B. subtilis* 168 cells grown in DSM. Primer extension was performed as described in Materials and Methods. The asterisk indicates the position of the primer extension products in the DNA sequence lying upstream of *yt**kD* (Fig. 1). The 5' end of the *yt**kD* 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. The results are representative and were performed at least twice. (A) Primer extension product located 80 to 81 bp upstream of the start codon of *yt**kD*. (B) Primer extension product located 31 to 32 bp upstream of the start codon of *yt**kD*.

5A, lane 1); such a product was also detected at lower levels in reactions performed with RNA isolated from cells at *T*₀ (Fig. 5A, lane 2). The second 5' end of *yt**kD* started 80 to 81 bp upstream of the first codon of *yt**kD*. Inspection of the sequences located upstream of this transcription start site allowed the identification of a second promoter that has conserved homology with those reported for σ^F -dependent genes (Fig. 6B). These results again support the conclusion that *yt**kD* is expressed during both vegetative growth and sporulation from the σ^A and σ^F promoters, respectively.

A *ytkD*-*lacZ* fusion is not induced by oxidative stress or during the SOS or σ^B general stress responses.** The strain *B. subtilis* PERM276 containing the *yt**kD*-*lacZ* fusion integrated at the *yt**kD* locus was used to investigate whether the *yt**kD* gene is induced as part of an oxidative stress regulon. Accordingly, strain *B. subtilis* PERM276 was grown to the mid-exponential phase and treated for 1 h with either paraquat (10 μ M) or hydrogen peroxide (200 μ M). The results (data not shown) revealed that no transcription induction occurred following the

treatment of the bacteria with these two oxidative stress-inducing chemicals.

Similarly, it has been shown that the expression of several genes whose products are putatively involved in mounting a general cellular response to conditions that promote a non-growing or starving state is under the control of the σ^B stress regulon (24, 25, 61). σ^B -dependent stress genes are strongly induced by heat, salt, acid, or ethanol as well as by energy depletion (24). We investigated whether *yt**kD* is part of the σ^B stress regulon by treating exponentially growing cells of the strain *B. subtilis* PERM276 with either sodium chloride (4%) or ethanol (4%) or heating the culture to 48°C. The results demonstrated that none of the stress conditions utilized was able to induce (during a period from 15 to 120 min) expression of the *yt**kD*-*lacZ* fusion (data not shown), suggesting that *yt**kD* is not part of the σ^B stress regulon. In agreement with this conclusion, our results showed that in a *B. subtilis* *sigB* mutant, the *yt**kD*-*lacZ* fusion followed a temporal pattern of expression similar to that observed in *B. subtilis* PERM276 (data not shown), reinforcing the idea that the transcription of *yt**kD* is not regulated by E(sig)B.

Additionally, we investigated whether *yt**kD* is part of the global SOS response (35), a gene circuitry that controls the expression of genes involved in DNA repair such as the *uvrA*, *C* (also termed *dinA*) and *recA* genes (36, 47). The SOS response in *B. subtilis* is induced following the introduction of certain types of damage into the chromosomal DNA and by the development of the physiological state of competence (37). Thus, the *B. subtilis* strain PERM276 was grown to the exponential phase and then treated with the DNA-damaging agent mitomycin C to a final concentration of 0.5 μ g/ml. After 1 h of mitomycin C treatment, the levels of β -galactosidase of the strain *B. subtilis* PERM 276 were not significantly raised above those expressed by the untreated control. On the contrary, the levels of β -galactosidase of a *recA*-*lacZ* fusion-containing *B. subtilis* strain were induced by mitomycin C treatment around 11 times above those expressed by the untreated control (data not shown).

DISCUSSION

Proteins containing the MutT motif are widely distributed among several species; however, not all of them catalyze the

FIG. 6. (A) Comparison of the consensus E(sig)A (23) promoter sequence (top line) with one of the putative promoter sequences (*P*_A) lying upstream of *yt**kD* (bottom line). (B) Comparison of the consensus E(sig)F (24) promoter sequence (top line) with the second putative promoter sequence (*P*_F) located upstream of *yt**kD*. Conserved (underlined) bases in E(sig)F-type promoters (24). H, A or C; R, A or G; X, A or T (24).

splitting of 8-oxo-dGTP (21, 45). This is best exemplified by *ytqG* (*nudF*) of *B. subtilis*, which despite encoding a MutT homolog, shows specificity to hydrolyze the diphosphate linkage of ADP-ribose (18). Therefore, the demonstration that a purified His₆-YtkD protein possessed the ability to catalyze the degradation of both 8-oxo-dGTP and 8-oxo-GTP (Table 2) revealed for the first time that *B. subtilis* possesses this type of antimutator protein to sanitize its nucleotide pools. In support of this conclusion, *ytqD* was able to genetically complement the mutator phenotype of an *E. coli mutT* mutant. It is relevant to point out that the level of complementation of the *E. coli mutT* strain with *ytqD* was similar to that obtained when the mutator phenotype of this strain was complemented with the cDNA of the human MutT homolog (*MTH1*) (58). As mentioned above, there is a high level of identity between YtkD, MutT, and MTH1 in the absolutely conserved residues of the MutT box (Fig. 1B) (23). Therefore, the variations of the level of complementation of *E. coli* SB3 with either *mutT*, *ytqD*, or *MTH1* most probably are the result of amino acid divergences lying out of the MutT boxes of these proteins. Thus, the ability of *ytqD* to genetically complement the DNA repair deficiency of the *mutT* *E. coli* mutant strongly suggests that its product confers protection to cells against the mutagenic effects of 8-oxo-dGTP and 8-oxo-GTP. Therefore, YtkD represents the first MutT homolog of *B. subtilis* with a demonstrated biochemical and physiological function, and as such, we propose to name it MutTA. It remains to be investigated whether MutT, YvcI, and YjhV, the other putative *B. subtilis* MutT homologs, encode true 8-oxo-dGTPases.

Upon finding that YtkD of *B. subtilis* possesses an activity involved in sanitizing the oxidized nucleotide pools, it was of interest to determine how the expression of *ytqD* is regulated by *B. subtilis* during vegetative growth and sporulation. Thus, a *ytqD-lacZ* fusion integrated by Campbell-type recombination into the *ytqD* locus of *B. subtilis* revealed that the transcription of *ytqD* is activated not only during vegetative growth but also during the first steps of sporulation. *ytqD* mRNAs were detected during both developmental stages, suggesting that *ytqD* is transcribed by the sequential action of RNA polymerases containing the σ^A and σ^F factors, respectively. In agreement with this suggestion, the spore-associated expression was almost completely abolished in a *sigF* genetic background but was not in a *B. subtilis* strain lacking a functional *sigG* gene.

In vivo mapping of the 5' ends of *ytqD* messengers expressed during vegetative growth allowed the identification of a putative promoter which showed to hold a significant degree of conserved homology with σ^A/σ^{70} -type promoters supporting the conclusion that the vegetative growth-associated transcription of *ytqD* occurs from a promoter that is recognized by σ^A RNA polymerase.

While there are a number of genes that belong to the σ^G regulon (24, 44, 53), there are many fewer genes that are known to be under σ^F control (4, 24). Consequently, the characteristics of the promoters that control the expression of σ^F -dependent genes are less well known. Experimental evidence detailed above indicated that the sporulation-associated expression of *ytqD* was dependent on σ^F RNA polymerase. A major extension product located 80 to 81 bp upstream of the putative *ytqD* start codon was amplified from RNA samples obtained from cells at *T*₅. The sequences that preceded this

putative transcriptional start site revealed the existence of a promoter with homology to the suggested consensus sequence of σ^F promoters (24). Although the -10 region was relatively far from the mapped 5' end of *ytqD*, it demonstrated conservation of 7 of the 9 consensus bases, including 2 characteristic guanines exclusively found in true σ^F -dependent promoters (4, 24). Furthermore, the -35 region that conserved 3 of the 5 σ^F consensus bases was shown to be separated from the -10 region by a 15-bp-long spacer, which is typical in these promoters (4, 24). Therefore, these results, together with the demonstrated forespore-specific expression and σ^F dependence of *ytqD*, strongly support the conclusion that *ytqD* is a new member of the σ^F regulon.

In the chromosome of *B. subtilis*, *ytqD* is preceded by an ORF termed *ytqC* encoding a putative autolytic amidase, and both genes are separated by an intergenic region. The lack of a transcriptional terminator in the 298-bp-long intergenic region is suggestive that genes are cotranscribed. However, the results of primer extension experiments, in combination with the detection of *ytqD* messengers of around 0.5 kb, indicate that *ytqD* is transcribed in a temporal manner from two different promoters located in the intergenic region between *ytqC* and *ytqD*.

B. subtilis displays an adaptive response to H₂O₂ that includes induction of *katA*, *ahpCF*, *mrgA*, and the *hemA* operon (3, 7, 9, 13, 17). Furthermore, it has been demonstrated that this response is regulated by PerR, a Fur homolog (10, 26). While the function of YtkD is associated with preventing the mutagenic effects of oxidized dNTPs, specific stress induction of the transcription of this gene does not appear to occur, since neither H₂O₂ nor paraquat treatment affected the levels of the gene product produced. Moreover, experimental evidence described here revealed that transcription of *ytqD* is not activated under conditions that promote the σ^B stress response (24). Likewise, the lack of induction of β -galactosidase in the *ytqD-lacZ* fusion strain following treatment by the DNA-damaging agent mitomycin C revealed that *ytqD* is not under the control of the SOS regulon (14, 62).

In addition to the transcriptional regulation of *ytqD* described in this paper, results of a proteomic study revealed that the synthesis of YtkD was induced under anaerobic growth, by nitrate respiration (15). The physiological relevance of these results remains to be established. In any case, the expression of the *ytqD* gene in the forespore raises the question as to what is the role played by this protein not only during developing of the sporulating cell but also in dormant spores. Clearly it is possible that a sanitizing role of YtkD would be inoperative in dormant spores, due in large part to their lack of metabolism and inability to perform DNA synthesis and transcription (54). Moreover, most of the enzymes existing in the spore core are believed to be in an inactive state (12, 54). On the other hand, it must be pointed out that production of ROS may well be exacerbated in germinating spores as a result of hydration of the spore cores and the triggering of metabolism. Thus, spore-specific protective enzymes might play an essential role in counteracting the effects of oxidative stress during spore germination and/or outgrowth. In support of this contention, the spore-specific catalase KatX was demonstrated to be essential for H₂O₂ resistance during spore germination (4). Accordingly, our laboratory is investigating the physiological role(s) played

by YtkD in the survival of vegetative cells of *B. subtilis* as well as a possible protective role of this enzyme against oxidative stress in the developing spore and/or during spore germination.

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