

Role of the Nfo and ExoA Apurinic/Apyrimidinic Endonucleases in Repair of DNA Damage during Outgrowth of *Bacillus subtilis* Spores[∇]

Juan R. Ibarra,¹ Alma D. Orozco,¹ Juan A. Rojas,¹ Karina López,¹ Peter Setlow,²
Ronald E. Yasbin,³ and Mario Pedraza-Reyes^{1*}

*Institute for Research in Experimental Biology, Faculty of Chemistry, University of Guanajuato, P.O. Box 187, Guanajuato, Gto. 36050, Mexico*¹; *Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06032*²; and *Department of Biology, University of Nevada-Las Vegas, 4505 Maryland Parkway, Box 454001, Las Vegas, Nevada 89154-400*³

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Germination and outgrowth are critical steps for returning *Bacillus subtilis* spores to life. However, oxidative stress due to full hydration of the spore core during germination and activation of metabolism in spore outgrowth may generate oxidative DNA damage that in many species is processed by apurinic/apyrimidinic (AP) endonucleases. *B. subtilis* spores possess two AP endonucleases, Nfo and ExoA; the outgrowth of spores lacking both of these enzymes was slowed, and the spores had an elevated mutation frequency, suggesting that these enzymes repair DNA lesions induced by oxidative stress during spore germination and outgrowth. Addition of H₂O₂ also slowed the outgrowth of *nfo* *exoA* spores and increased the mutation frequency, and *nfo* and *exoA* mutations slowed the outgrowth of spores deficient in either RecA, nucleotide excision repair (NER), or the DNA-protective α/β -type small acid-soluble spore proteins (SASP). These results suggest that α/β -type SASP protect DNA of germinating spores against damage that can be repaired by Nfo and ExoA, which is generated either spontaneously or promoted by addition of H₂O₂. The contribution of RecA and Nfo/ExoA was similar to but greater than that of NER in repair of DNA damage generated during spore germination and outgrowth. However, *nfo* and *exoA* mutations increased the spontaneous mutation frequencies of outgrown spores lacking *uvrA* or *recA* to about the same extent, suggesting that DNA lesions generated during spore germination and outgrowth are processed by Nfo/ExoA in combination with NER and/or RecA. These results suggest that Nfo/ExoA, RecA, the NER system, and α/β -type SASP all contribute to the repair of and/or protection against oxidative damage of DNA in germinating and outgrowing spores.

Due to their ability to survive during long periods of dormancy, spores of *Bacillus subtilis* are an excellent model system in which to study the consequences of exposure to environmental factors that can cause DNA damage. Physical and chemical factors, including UV-A and -B from sunlight, high temperatures, desiccation, and oxidizing chemicals, such as hydrogen peroxide, have the potential to cause damage to dormant spore DNA (13, 15). However, spores of the genus *Bacillus* counter these potential damaging agents with a number of factors to maintain the integrity of the genome. These factors include (i) the spore coats, (ii) the low water content in the spore core, (iii) the low permeability of the spore's inner membrane to hydrophilic small molecules, and (iv) the saturation of spore DNA with α/β -type small acid-soluble spore proteins (SASP) (13, 28, 29, 31). The α/β -type SASP play the most direct role in protecting spore DNA from a variety of types of damage, including depurination-depyrimidination and hydroxyl radical-induced backbone cleavage. As a consequence, these proteins make major contributions to spore resistance to heat and oxidizing agents (13, 31). The α/β -type SASP are also a major factor in the high resistance of *B. subtilis*

spores to UV light (13, 14, 28, 29, 31). However, these DNA binding proteins do not protect DNA against base alkylation (27).

Despite the existence of mechanisms that prevent DNA damage during long periods of dormancy and upon exposure to environmental stresses, spores can accumulate potentially lethal and mutagenic DNA damage (13, 28, 29, 31). Such damage may include strand breaks, as well as apurinic-apyrimidinic (AP) sites. The latter lesions are processed by AP endonucleases, which are important components of the base excision repair pathway. *B. subtilis* spores contain two AP endonucleases, Nfo and ExoA, and a recent report indicated that these enzymes are important in protecting the dormant spore from treatments that promote base loss and strand breaks in DNA (20).

Since spores are metabolically dormant and enzymes in the spore core, the site of spore DNA, are inactive, there can be no DNA repair in the dormant spore. However, spores can "return to life" during the process of germination (17, 30), which is triggered when specific germinants, generally amino acids or sugars, are sensed by receptors in the spore's inner membrane. This receptor-germinant interaction triggers germination events, including the release of dipicolinic acid and divalent cations from the spore core, hydrolysis of the spore cortex peptidoglycan, and uptake of water into the spore core to levels comparable to those in growing cells. The completion of spore germination (in particular, full core rehydration) then allows resumption of enzyme action in the spore core and the

* Corresponding author. Mailing address: Institute for Research in Experimental Biology, Building L, Faculty of Chemistry, University of Guanajuato, Noria Alta S/N, Guanajuato, 36050 Gto., Mexico. Phone: (473) 73 2 00 06, ext. 8161. Fax: (473) 73 2 00 06, ext. 8153. E-mail: pedrama@quijote.ugto.mx.

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype and description	Source and/or reference
<i>B. subtilis</i> strains		
168	Wild type, <i>trp</i>	Laboratory stock
PS832	Wild type, <i>trp</i> ⁺ revertant of strain 168	Laboratory stock
PS356 ^a	Δ <i>sspA</i> Δ <i>sspB</i> $\alpha^- \beta^-$	10
PERM450 ^a	Δ <i>sspA</i> Δ <i>sspB</i> Δ <i>exoA::tet</i> Δ <i>nfo::neo</i> $\alpha^- \beta^-$ Neo ^r Tet ^r	20
PERM452 ^a	Δ <i>exoA::tet</i> Tet ^r	20
PERM453 ^a	Δ <i>nfo::neo</i> Neo ^r	20
PERM454 ^a	Δ <i>exoA::tet</i> Δ <i>nfo::neo</i> Neo ^r Tet ^r	20
YB300	<i>recA</i> Cm ^r	R. Yasbin (36)
PERM662 ^a	<i>recA</i> Cm ^r	(YB300 → PS832) ^b
PERM663 ^a	<i>recA</i> Δ <i>exoA::tet</i> Cm ^r Tet ^r	(pPERM374 → PS599) ^c
PERM665 ^a	<i>recA</i> Δ <i>exoA::tet</i> Δ <i>nfo::neo</i> Cm ^r Tet ^r Neo ^r	(pPERM337 → PERM663) ^c
PS599 ^a	<i>uvrA42</i> <i>trp</i> ⁺	P. Setlow
PERM515 ^a	<i>uvrA</i> Δ <i>exoA::tet</i> Tet ^r	(pPERM374 → PS599) ^c
PERM517 ^a	<i>uvrA</i> Δ <i>exoA::tet</i> Δ <i>nfo::neo</i> Tet ^r Neo ^r	(pPERM337 → PERM515) ^c
Plasmids		
pPERM337	1.3-kb SmaI-SmaI fragment containing a Neo ^r cassette inserted into the NaeI site of <i>nfo</i> in pPERM282; Amp ^r Neo ^r	20
pPERM374	469-bp XbaI-BamHI fragment (3' region of <i>exoA</i>) and 537-bp EcoRI-HindIII fragment (5' region of <i>exoA</i>) flanking the tetracycline gene of pDG1515; Amp ^r Tet rd	20

^a The background for this strain is PS832.

^b Chromosomal DNA from the strain to the left of the arrow was used to transform the strain to the right of the arrow.

^c DNA of the plasmid to the left of the arrow was used to transform the strain to the right of the arrow.

^d See reference 6.

initiation of spore outgrowth that eventually converts the germinated spore into a growing cell (17). A crucial event early in spore outgrowth is the degradation of the α/β -type SASP by a specific spore protease; this degradation frees up spore DNA for transcription and eventually for replication (28, 32). The free amino acids produced in this proteolysis also support much of the energy metabolism that begins early in spore outgrowth.

While dormant spore DNA is well protected against damage, as noted above, the protective mechanisms are lost upon completion of spore germination and early in spore outgrowth, when the core water content increases and α/β -type SASP are degraded. Aerobic metabolism resumes only early in spore outgrowth and can lead to the production of reactive oxygen species (ROS). These ROS can generate a variety of types of DNA damage, including AP sites, several different base modifications, sugar damage, and single- and double-strand breaks (5, 19, 22, 34). As a consequence, DNA repair proteins packaged in the spore may be extremely important in spore outgrowth not only to repair damage that accumulated during spore dormancy but also to repair damage generated during spore germination and outgrowth. Indeed, a spore-specific catalase, KatX, plays a major role in protecting spores against oxidative stress during spore germination and outgrowth (1).

As noted above, germination and outgrowth are critical steps in the return of *B. subtilis* spores to vegetative growth, and during these processes spores may suddenly face an increase in ROS-induced DNA damage. Given the potential role of the AP endonucleases Nfo and ExoA in repair of such DNA damage (20), we investigated of the effects of *nfo* and *exoA* mutations on *B. subtilis* spore germination and outgrowth with and without an added oxidative stress agent, and in this paper we report the results of this investigation. We also examined the effects of loss of the majority of α/β -type SASP or other

DNA repair proteins alone or in combination with *nfo* *exoA* mutations on spore germination and outgrowth, as well as on mutagenesis during these periods of development.

MATERIALS AND METHODS

Bacterial strains and spore preparation. All *B. subtilis* strains used in this work except YB300 were derived from strain PS832, a prototrophic derivative of strain 168, and they are listed in Table 1 along with the plasmids used. The wild-type strain referred to below is PS832, unless noted otherwise. The growth medium used routinely was Luria-Bertani (LB) medium (11). When appropriate, ampicillin (100 μ g/ml), chloramphenicol (5 μ g/ml), neomycin (10 μ g/ml), or tetracycline (10 μ g/ml) was added to the medium. Liquid cultures were incubated at 37°C with vigorous aeration. Cultures on solid media were also grown at 37°C.

Spores of all strains were prepared at 37°C on 2× SG medium agar plates without antibiotics, and spores were harvested, cleaned, and stored as described previously (16). All dormant spore preparations used in this work were free ($\geq 98\%$) of growing cells, germinated spores, and cell debris, as determined by phase-contrast microscopy.

Spore germination and outgrowth. Unless otherwise noted, spore germination and outgrowth were performed in 25 ml of 2× SG medium supplemented with 10 mM L-alanine. Spores in water were first heat shocked for 30 min at 70°C, cooled on ice, and inoculated into germination medium at 37°C to obtain an initial optical density at 600 nm (OD₆₀₀) of ~0.5. Where noted below, H₂O₂ (0.2 mM) was added after most spore germination had taken place (~15 min after the initiation of spore germination). The OD₆₀₀ of cultures were monitored with a Pharmacia Ultrospec 2000 spectrophotometer, and the values were plotted as a fraction of the initial OD₆₀₀ (OD₆₀₀ at time *t*/initial OD₆₀₀) versus time.

Genetic and molecular biology techniques. Chromosomal DNA was isolated from *B. subtilis*, and competent *B. subtilis* cells were prepared and transformed with plasmid or chromosomal DNA as described previously (2, 4). For medium-scale preparation and purification of plasmid DNA commercial ion-exchange columns were used according to the instructions of the supplier (Qiagen, Inc., Valencia, CA).

Construction of mutant strains. Plasmids pPERM337 and pPERM374 (20) were used to transform *B. subtilis* strains PS599 (*uvrA*) and PERM662 (*recA*) to resistance to the appropriate antibiotics, which generated strains PERM517 (*uvrA nfo* *exoA*) and PERM665 (*recA nfo* *exoA*). The double recombination events leading to inactivation of the appropriate genes were confirmed by PCR (data not shown). Strains PERM454 (*nfo* *exoA*) and PERM450 ($\alpha^- \beta^-$ *nfo* *exoA*) have been described previously (20). A *recA* derivative of PS832 was generated

by transformation to Cm^r with chromosomal DNA (1 μ g) from strain YB300 (36). The *recA* phenotype of Cm^r transformants was confirmed by sensitivity to mitomycin C, as described previously (3). The *exoA* mutant was generated by deleting $\sim 20\%$ of the *exoA* open reading frame and replacing it with a tetracycline resistance cassette; the *nfo* mutant was constructed by inserting a neomycin resistance cassette in the middle of the *nfo* open reading frame (20).

Analysis of spontaneous mutation frequencies. Spontaneous mutations to rifampin resistance (Rif^r) in outgrown spore cultures were determined as follows. Spores were germinated in $2\times$ SG medium supplemented with 10 mM L-alanine as described above. Aliquots removed from cultures 180 min after inoculation of spores into germination medium were spread on six LB medium plates containing 10 μ g/ml rifampin, and Rif^r colonies were counted after 1 day of incubation at 37°C . The number of cells used to calculate the frequency of mutation to Rif^r was determined by plating aliquots of appropriate dilutions on LB medium plates without rifampin and incubating the plates for 24 to 48 h at 37°C . Mutations to Rif^r in growing cells were determined as follows. Overnight cultures of each strain were inoculated into flasks containing LB medium to an OD_{600} of 0.5, each culture was divided in half, and the two halves were transferred to different flasks. One of the cultures was left untreated, and 0.2 mM H_2O_2 was added to the other. Both flasks were shaken at 37°C for 180 min, and aliquots were processed to determine the frequency of mutation to Rif^r as described above. These experiments were repeated at least twice.

RESULTS

Germination and outgrowth of spores lacking Nfo and ExoA. We first investigated how mutations in *nfo* or *exoA* or in both genes affected the germination and outgrowth of *B. subtilis* spores by inoculating *B. subtilis* wild-type, *nfo*, *exoA*, and *nfo* *exoA* spores into germination medium and monitoring the germination and outgrowth by determining the OD_{600} . Compared with wild-type spores, *nfo* and *exoA* spores exhibited slower outgrowth, although they exhibited essentially identical rates of germination when germination was judged by determining the decrease in the OD_{600} of spore cultures shortly after addition of spores to germination medium (data not shown). Note that the initial decrease in the OD_{600} was due to both release of dipicolinic acid and full hydration of the spore core, major events in spore germination. The germination rate of spores lacking both Nfo and ExoA was similar to that of wild-type spores, but the return to vegetative growth (outgrowth) was significantly slower for the *nfo* *exoA* spores (Fig. 1A). The latter effect may have been due at least in part to DNA damage as the outgrown *nfo* *exoA* spores accumulated ~ 10 -fold more mutations than the outgrown wild-type spores or growing *nfo* *exoA* cells (Fig. 2). Thus, a tentative conclusion from these results is that significant damage occurs in the DNA of germinating and outgrowing spores, perhaps generated by oxidative stress, and that Nfo and ExoA are required to eliminate the lesions. To further investigate this idea, *nfo* *exoA* spores were outgrown with 0.2 mM H_2O_2 added ~ 15 min after the initiation of germination, when most spore germination was complete, as shown by the decrease in the OD_{600} (Fig. 1B). Although the H_2O_2 slowed the outgrowth of wild-type spores, the effect was greater with spores lacking Nfo and ExoA. In addition, the mutation frequency in outgrown *nfo* *exoA* spores was ~ 10 -fold higher than that in outgrown wild-type spores treated with H_2O_2 (Fig. 3). However, H_2O_2 was mutagenic for both wild-type and *nfo* *exoA* cells and spores, as shown by the ~ 10 -fold-higher mutation frequencies of the cultures treated with H_2O_2 .

Effects of *nfo* and *exoA* mutations on germination and outgrowth of spores lacking α/β -type SASP. The main factor protecting dormant spore DNA from damage is the α/β -type

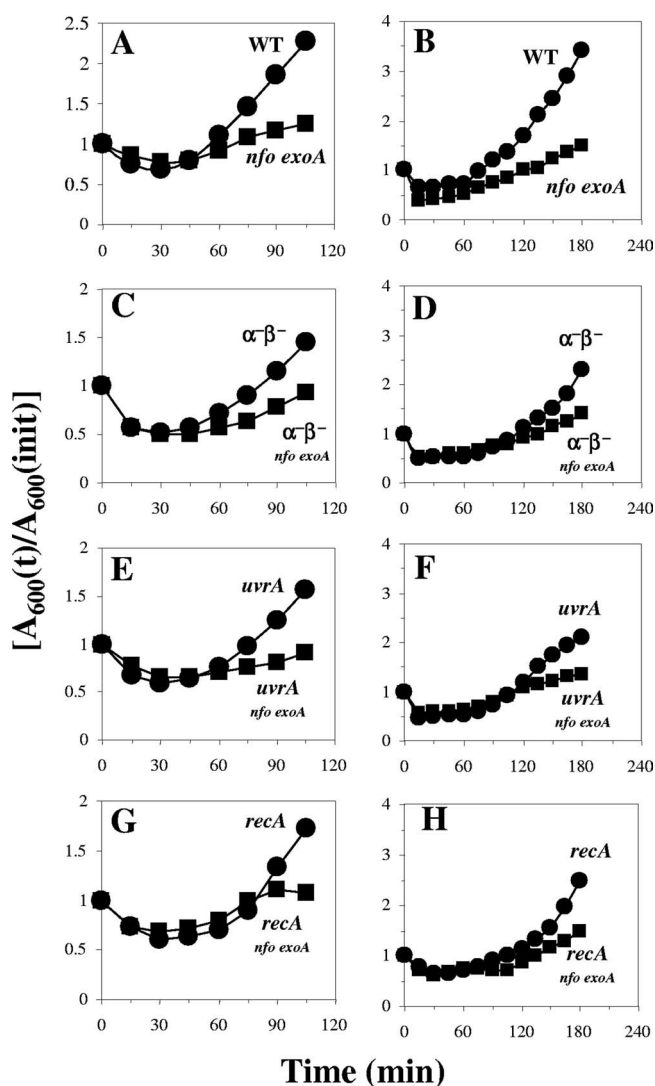


FIG. 1. Germination and outgrowth of spores of different strains. Dormant spores of the wild-type (●) and *nfo* *exoA* (■) strains (A and B), $\alpha^- \beta^-$ (●) and $\alpha^- \beta^-$ *nfo* *exoA* (■) strains (C and D), *uvrA* (●) and *uvrA* *nfo* *exoA* (■) strains (E and F), and *recA* (●) and *recA* *nfo* *exoA* (■) strains (G and H) were heat shocked and germinated without (A, C, E, and G) or with (B, D, F, and H) 0.2 mM H_2O_2 added ~ 15 min after the start of germination, and spore germination and outgrowth were measured by monitoring the OD_{600} of the cultures as described in Materials and Methods. The results are representative, and the experiment was performed at least three times with different lots of spores, all of which produced similar results. WT, wild type.

SASP, as spores lacking the majority of these proteins (termed $\alpha^- \beta^-$ spores) are much more sensitive to a variety of DNA-damaging treatments (13, 23, 24). Perhaps not surprisingly, the *nfo* *exoA* mutations slowed the outgrowth of $\alpha^- \beta^-$ spores, although they had little or no effect on spore germination (Fig. 1C). The mutation frequency of outgrown $\alpha^- \beta^-$ spores was about seven times higher than that of outgrown wild-type spores, and the value was increased two- to threefold by the *nfo* *exoA* mutations (Fig. 2). These results suggest that α/β -type SASP may also play an important role in protecting DNA against damage generated during spore germination and out-

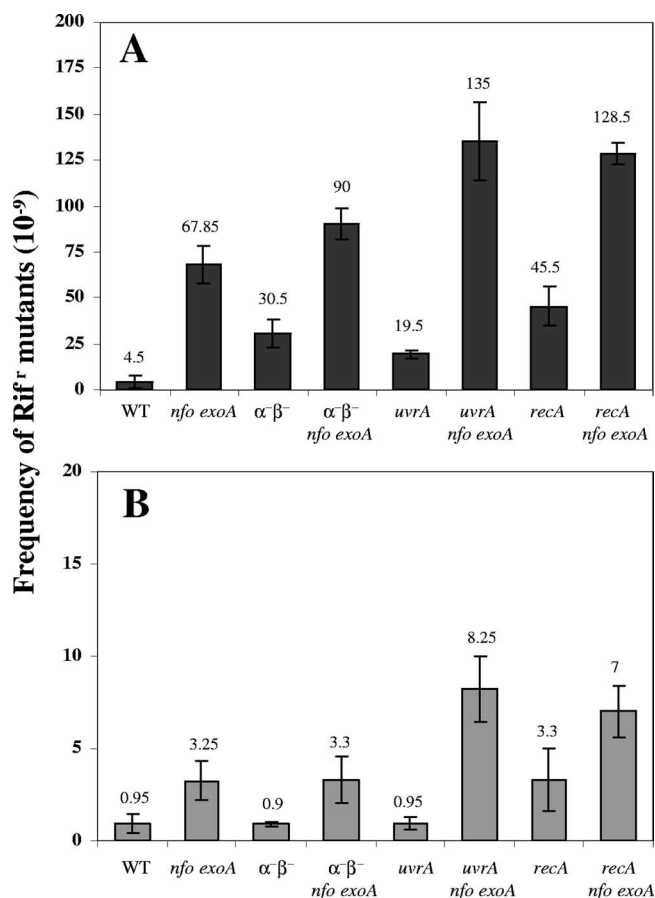


FIG. 2. Mutation frequencies in outgrown spores and growing cells of different strains. (A) Dormant spores of different strains were heat shocked and germinated, aliquots were taken after 180 min, and frequencies of mutation to Rif^r were determined as described in Materials and Methods. (B) Overnight cultures of growing cells of different strains were inoculated into LB medium, and after 180 min of growth the frequencies of mutation to Rif^r were determined as described in Materials and Methods. The values are averages \pm standard deviations for duplicate determinations in two separate experiments (with different cultures or lots of spores). WT, wild type.

growth. H₂O₂ also significantly slowed the outgrowth of α⁻β⁻ spores, and this effect was increased when Nfo and ExoA were also absent (Fig. 1D). The mutation frequencies in outgrown H₂O₂-treated α⁻β⁻ spores and α⁻β⁻ *nfo* *exoA* spores were also ~4- and 10-fold higher than that in outgrown H₂O₂-treated spores that contained α/β-type SASP, respectively (Fig. 3).

Effects of *nfo* and *exoA* mutations on germination and outgrowth of spores lacking either recombination repair or NER. The nucleotide excision repair (NER) pathway plays an important role in protecting *B. subtilis* against a number of agents that cause DNA damage (39, 40, 41). Consequently, mutations in genes encoding NER components, particularly *uvrA* or *uvrC*, make *B. subtilis* cells more sensitive to several factors, including UV-C light, mitomycin C, and H₂O₂ (40, 41). In *B. subtilis* the expression of genes involved in DNA repair, including some genes of the NER pathway, is induced by DNA damage and during competence (3, 8, 9). This response, which may well be triggered by single-stranded DNA, is called the SOS re-

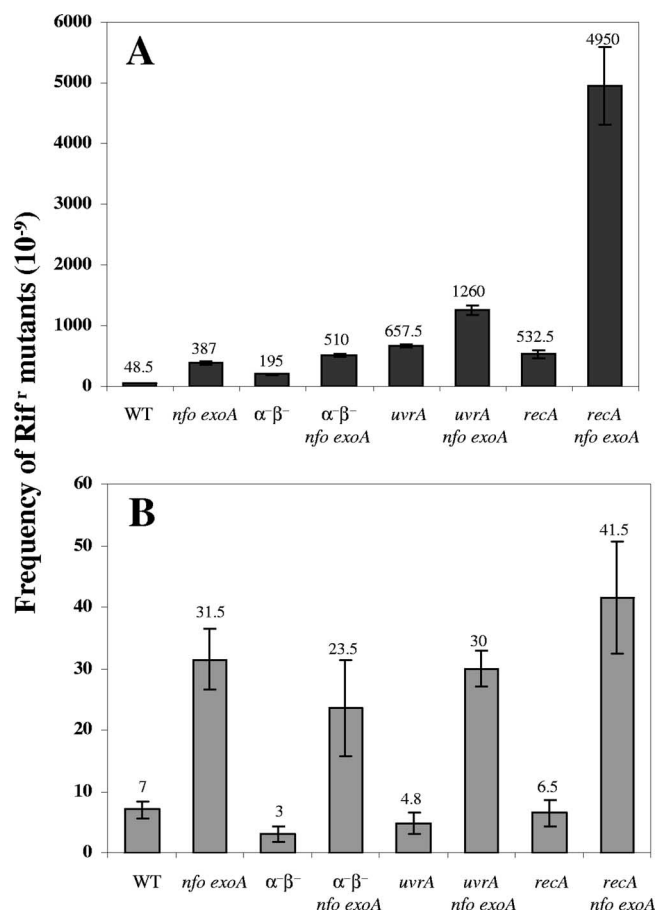


FIG. 3. Mutation frequencies of outgrown spores and growing cells of different strains treated with H₂O₂. (A) Dormant spores of different strains were heat shocked and germinated, 0.2 mM H₂O₂ was added 15 min after the start of spore germination, aliquots were taken after 180 min of incubation, and frequencies of mutation to Rif^r were determined as described in Materials and Methods. (B) Overnight cultures of growing cells of different strains were inoculated into LB medium, 0.2 mM H₂O₂ was added, and after 180 min of growth frequencies of mutation to Rif^r were determined as described in Materials and Methods. The values are averages \pm standard deviations for duplicate determinations in two separate experiments (with different cultures or lots of spores). WT, wild type.

sponse and is controlled by RecA and the transcriptional repressor DinR (18, 38).

Since UvrA and RecA play important roles in protecting *B. subtilis* from DNA damage, we investigated how *nfo* and *exoA* mutations in either a *uvrA* or *recA* genetic background affected spore germination and outgrowth. The absence of Nfo and ExoA in *uvrA* and *recA* spores again resulted in a slow-outgrowth phenotype (Fig. 1E and 1G). In addition, outgrown *uvrA* and *recA* spores had a higher mutation frequency than outgrown wild-type spores, and this phenotype was exacerbated when Nfo and ExoA were absent in either genetic background (Fig. 2).

The rate of outgrowth of *uvrA* and *recA* spores was also decreased by addition of H₂O₂, and this effect was greater with the *uvrA* *nfo* *exoA* and *recA* *nfo* *exoA* spores (Fig. 1F and 1H). The H₂O₂ most probably damaged the DNA of the outgrown

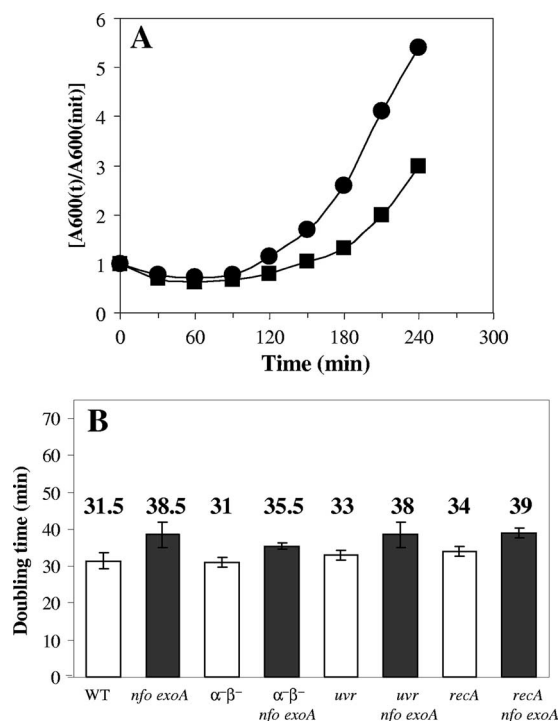


FIG. 4. Spore germination and outgrowth without prior heat activation and doubling times of different strains. (A) Wild-type (●) and *nfo exoA* (■) dormant spores were germinated without a prior heat shock, and spore germination and outgrowth were measured by monitoring the OD₆₀₀ of the cultures as described in Materials and Methods. The results are representative, and the experiment was performed at least two times with different lots of spores, all of which produced similar results. (B) Overnight cultures of various strains were inoculated into 25 ml of LB medium, the cultures were shaken at 37°C, and the OD₆₀₀ of the cultures were monitored. The doubling times are the averages \pm standard deviations for values obtained from two different cultures. WT, wild type.

uvrA and *recA* spores, since these outgrown spores had ~10-fold-higher mutation frequencies than outgrown wild-type spores (Fig. 3). The absence of Nfo and ExoA also resulted in a dramatic increase in the mutation frequency of outgrown H₂O₂-treated *recA* spores (Fig. 3). However, outgrown H₂O₂-treated *uvrA nfo exoA* spores had a mutation frequency that was only about twofold higher than that of outgrown H₂O₂-treated *uvrA* spores (Fig. 3).

Effect of *nfo* and *exoA* mutations during spore germination and outgrowth is not due to DNA damage generated during storage or activation of spores. While the effects of Nfo and ExoA on spore germination and outgrowth were dramatic, particularly in minimizing mutagenic DNA damage, it was possible that the damage was generated not during spore germination or outgrowth but during the heat treatment at 70°C used to activate spores prior to germination. However, when wild-type and *nfo exoA* spores were germinated without prior heat activation, the outgrowth of the *nfo exoA* spores was still slower than the outgrowth of wild-type spores (Fig. 4A). However, spore germination and outgrowth, particularly germination, were more asynchronous and thus slower without prior heat activation (compare Fig. 4A and 1A).

A second possible explanation for differences in outgrowth

of spores with and without different combinations of DNA repair or protective genes is that the mutations decrease the rate of cell growth significantly, since such a decrease might slow spore outgrowth. However, while the *nfo exoA* mutations did increase the vegetative cell doubling times very slightly, this effect was minimal, and the other mutations had no effect on cell growth rates (Fig. 4B). Consequently, it appears very unlikely that this was the cause of the slow outgrowth of the spores of the various strains.

A third possible explanation for the slow outgrowth of spores deficient in DNA repair or protection, as well as the accumulation of DNA damage in these spores, is that the DNA damage was not generated during spore germination and outgrowth but during spore preparation and storage. Indeed, it has been shown that $\alpha^- \beta^-$ spores can accumulate significant amounts of DNA damage during long-term storage (32). To examine this possibility, genomic DNA was isolated from dormant spores of the PS832 (wild type; Trp⁺), *nfo exoA*, $\alpha^- \beta^-$, and $\alpha^- \beta^-$ *nfo exoA* strains and was introduced by transformation into *B. subtilis* 168 (Trp⁻), and Rif^r and Rif^r Trp⁺ colonies were selected. The results of this experiment (Table 2) showed that the genomic DNA from the three types of mutant dormant spores increased the number of Rif^r Trp⁺ colonies only about twofold compared to the colonies generated by DNA from wild-type spores, and the numbers of Trp⁺ colonies/mg of transforming DNA were essentially identical for all the DNAs from strains with PS832 backgrounds (data not shown).

Effects of *nfo* and *exoA* mutations on vegetative cells deficient in recombination repair or NER. As noted above, the *nfo exoA* deletions alone or combined with either *uvrA* or *recA* mutations delayed the return of outgrowing spores to vegetative growth and increased the outgrowing spores' susceptibility to H₂O₂. In addition, these outgrown spores had a high mutation frequency, and the frequency was higher for the *recA nfo exoA* and *uvrA nfo exoA* spores (Fig. 2). UvrA and RecA are both involved in repairing DNA damage in vegetative cells (39, 40, 41), and *exoA* expression, but not *nfo* expression, takes place in growing cells (20, 33). Therefore, we investigated whether the requirement for these proteins was different during spore outgrowth and exponential growth. As noted above (Fig. 4B), there were no major differences in the doubling

TABLE 2. Transformation of *B. subtilis* 168 from Trp⁻ to Rif^r Trp⁺ using genomic DNA from dormant spores of various strains

Strain	Frequency of Rif ^r mutants (10 ⁻⁹) ^c	No. of Rif ^r Trp ⁺ colonies/100 Rif ^r colonies ^d
168 Trp ^{-a}	0.83 \pm 0.16	1.5 \pm 0.7
PS832 Trp ⁺ \rightarrow 168 Trp ^{-b}	0.85 \pm 0.21	4.5 \pm 1.4
<i>nfo exoA</i> \rightarrow 168 Trp ^{-b}	0.91 \pm 0.14	9.5 \pm 2.1
$\alpha^- \beta^- \rightarrow$ 168 Trp ^{-b}	0.94 \pm 0.05	9.0 \pm 1.4
$\alpha^- \beta^-$ <i>nfo exoA</i> \rightarrow 168 Trp ^{-b}	0.99 \pm 0.16	10.5 \pm 1.4

^a Untransformed strain.

^b Genomic DNA (1 μ g) from dormant spores of the strain to the left of the arrow was used to transform competent cells of the strain to the right of the arrow with selection for Rif^r.

^c Averages \pm standard deviations for two independent experiments.

^d One hundred Rif^r colonies from the two frequency experiments were transferred to agar plates containing 1 \times Spizizen's medium salts (35) supplemented with 0.5% glucose to screen for auxotrophy. The values are the averages \pm standard deviations for the two experiments.

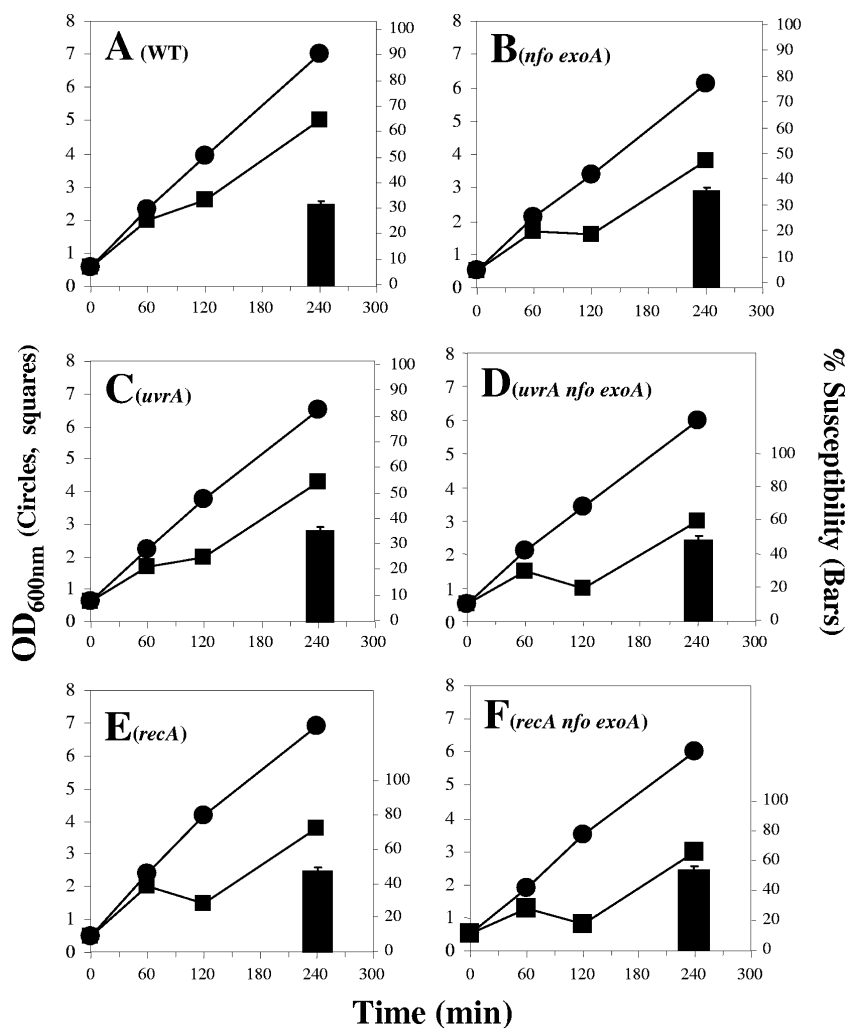


FIG. 5. Growth of different strains in the absence or presence of H₂O₂. Overnight cultures of the wild-type (A), *nfo exoA* (B), *uvrA* (C), *uvrA nfo exoA* (D), *recA* (E), and *recA nfo exoA* (F) strains were inoculated into 25 ml of LB medium and grown to an OD₆₀₀ of ~0.5. Each culture was divided into two subcultures; 0.2 mM H₂O₂ was added to one subculture (■), and nothing was added to the other subculture (●); and cell growth was monitored by determining the OD₆₀₀. The results are representative, and the experiments were performed at least two times with different cultures. The percentage of hydrogen peroxide susceptibility (bars) was calculated for each strain at 240 min by using the following equation: $(1 - \text{OD}_{600} \text{ of H}_2\text{O}_2\text{-treated culture} / \text{OD}_{600} \text{ of nontreated culture}) \times 100$. The values are the averages and standard deviations for duplicate determinations in two separate experiments. WT, wild type.

times of the wild-type, $\alpha^- \beta^-$, *uvrA*, and *recA* strains, although the *nfo exoA* mutations slightly increased the doubling times in the three genetic backgrounds. In addition, as previously described (20), cells lacking Nfo and ExoA were around 1.2 times more susceptible to H₂O₂ treatment than cells of the parental strain (Fig. 5A and B). Similar levels of susceptibility were obtained when $\alpha^- \beta^-$ cells and $\alpha^- \beta^- nfo exoA$ cells were treated with H₂O₂ during exponential growth (data not shown). On the other hand, after 240 min of growth, cells of *uvrA* and *recA* strains were around 5 and 16% more susceptible to H₂O₂ than wild-type cells, respectively, and this effect was increased around 13 and 7% in the *uvrA nfo exoA* and *recA nfo exoA* strains, respectively (Fig. 5C to F).

Exponentially growing cells of *nfo exo*, *recA nfo exoA*, and *uvrA nfo exoA* strains had a higher mutation frequency than their parental strains whether or not they were treated with H₂O₂ (Fig. 2, 3). However, in the absence of H₂O₂, the muta-

tion frequencies of the *nfo exo*, *recA nfo exoA*, *uvrA nfo exoA*, and parental strains were ≥ 10 -fold higher in outgrown spores than in growing cells, and in the presence of H₂O₂ the mutation frequencies were ≥ 100 -fold higher in outgrown spores than in growing cells of the same strains (Fig. 2 and 3).

DISCUSSION

The entrance of water into the spore core and the activation of aerobic metabolism in the outgrowing spore may generate DNA damage that is processed by the base excision repair pathway. Indeed, our results revealed that an absence of the AP endonucleases Nfo and ExoA in *B. subtilis* significantly delayed spore outgrowth. This phenotype appears to be probably due to DNA damage, since outgrown *nfo exoA* spores had a significantly higher mutation frequency than outgrown wild-type spores or growing *nfo exoA* or wild-type cells. The

slow-outgrowth phenotype of *nfo* *exoA* spores was not the result of DNA damage generated during heat activation prior to spore germination, although some of the DNA damage found in outgrown spores deficient in DNA repair or protection may have accumulated in dormant spores. However, normally, the amount of the latter damage seems to be small compared to the DNA damage generated in spore germination and outgrowth, as the mutation frequency in outgrown *nfo* *exoA* spores was ~20-fold higher than that in outgrown wild-type spores, while the dormant spore DNAs from these two types of spores exhibited only an ~2-fold difference in mutations when the DNAs were assayed by examining transformation. Consequently, these results support the notion that oxidative DNA damage occurs in the germinating and outgrowing spore and Nfo and ExoA have an important role in eliminating this type of damage. In addition, since wild-type and *nfo* *exoA* dormant spores were equally sensitive to H₂O₂ (20), it is reasonable to propose that (i) the DNA resistance to H₂O₂ is different in dormant spores and germinating and outgrowing spores and (ii) Nfo and ExoA are important in eliminating H₂O₂-induced DNA damage during spore outgrowth. The results described in this paper also implicate α/β -type SASP in protecting the germinating and outgrowing spores from DNA damage, since the mutation frequency was higher in outgrown $\alpha^- \beta^-$ spores than in outgrown wild-type spores and was significantly increased in $\alpha^- \beta^-$ spores by mutations in *nfo* and *exoA*. However, the resistance conferred by α/β -type SASP to dormant spores against several DNA-damaging factors is ultimately lost during spore outgrowth (17, 23, 24), and thus the protective effect postulated here would operate only until α/β -type SASP are degraded by the specific germination protease germination protease in outgrowth (21, 28). Indeed, spore resistance to UV radiation persists well after the initiation of spore germination (21). A recent study revealed that loss of ExoA and Nfo did not sensitize growing cells or wild-type or $\alpha^- \beta^-$ spores to H₂O₂ and *t*-butylhydroperoxide (20). However, our results showed that outgrowth of $\alpha^- \beta^-$ spores and $\alpha^- \beta^-$ *nfo* *exoA* spores was significantly slowed by H₂O₂ and that there was a concomitant increase in the mutation frequency of the outgrown spores compared with that of outgrown wild-type spores. Thus, in contrast to what has been observed for dormant spores, the DNA of germinating and outgrowing spores (this study) and the DNA of dormant spores lacking α/β -type SASP (24, 25, 27, 28) are evidently subject to damage by H₂O₂. A recent study showed that neither ExoA nor Nfo repaired the DNA damage induced by hydrogen peroxide in $\alpha^- \beta^-$ spores (20). In contrast, the current results implicate Nfo and ExoA in repair of H₂O₂-induced DNA damage during spore germination and outgrowth. Thus, it is possible that the DNA lesions induced by H₂O₂ during spore germination and outgrowth are different from those generated in dormant $\alpha^- \beta^-$ spores.

As noted above, RecA and UvrA are also involved in repairing spore DNA damage (26), and *recA* spores are more sensitive to UV-C and dry heat than wild-type spores (7, 12, 26). The results of this work support the idea that NER and RecA are also involved in repairing DNA damage that accumulates during spore germination and outgrowth. Analysis of the mutation frequencies in outgrown *nfo* *exoA*, *recA*, and *uvrA* spores revealed that RecA and Nfo/ExoA make similar con-

tributions to repair of DNA damage that accumulates during spore germination and outgrowth, while the contribution of NER is less. However, the *nfo* and *exoA* mutations increased the mutation frequencies of outgrown *uvrA* and *recA* spores to about the same level, suggesting that some DNA lesions generated during germination and outgrowth are processed by Nfo and ExoA, as well as by NER and RecA. Previous results also showed that a *recA* mutation renders $\alpha^- \beta^-$ spores less resistant to H₂O₂, suggesting that RecA is directly or indirectly involved in repairing H₂O₂-induced DNA lesions in these spores (26). In the present work, treatment of germinating and outgrowing *recA* spores with H₂O₂ revealed the importance of RecA and Nfo/ExoA in the repair of DNA damage induced by oxidative stress. Therefore, our results strongly suggest that much of the DNA damage caused by H₂O₂ during spore germination and outgrowth is processed by Nfo/ExoA and RecA.

Our results also showed that the *nfo* *exoA* mutations affected the growth rate of growing *uvrA* and *recA* cells slightly, and this effect was greater when cells were treated with H₂O₂. However, comparison of the mutation frequencies in growing cells and outgrown spores with and without H₂O₂ treatment suggested that the requirement for UvrA, RecA, and Nfo/ExoA for repair of oxidative DNA damage is greater in outgrowing spores than in growing cells.

Finally, although the *nfo* *exoA* mutations slowed the outgrowth of all spores tested, these mutations did not stop this process, suggesting that spores have mechanisms that allow outgrowth even with minimal DNA repair. On the other hand, the high levels of mutations observed in outgrown spores deficient in Nfo/ExoA and RecA either in the presence or in the absence of H₂O₂ suggest that, as previously proposed (27), DNA lesions can be either repaired or bypassed during spore outgrowth in an error-prone manner, perhaps with participation of DNA polymerase YqjH and/or DNA polymerase YqjW (37).

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