Role of Ribonucleotide Reductase in *Bacillus subtilis* Stress-Associated Mutagenesis

Karla Viridiana Castro-Cerritos,^a Ronald E. Yasbin,^c Eduardo A. Robleto,^b Mario Pedraza-Reyes^a

Department of Biology, Division of Natural and Exact Sciences, University of Guanajuato, Guanajuato, Mexico^a; School of Life Sciences, University of Nevada, Las Vegas, Nevada, USA^b; College of Arts and Sciences, University of Missouri—St. Louis, St. Louis, Missouri, USA^c

ABSTRACT The Gram-positive microorganism *Bacillus subtilis* relies on a single class Ib ribonucleotide reductase (RNR) to generate 2'-deoxyribonucleotides (dNDPs) for DNA replication and repair. In this work, we investigated the influence of RNR levels on *B. subtilis* stationary-phase-associated mutagenesis (SPM). Since RNR is essential in this bacterium, we engineered a conditional mutant of strain *B. subtilis* YB955 (*hisC952 metB5 leu427*) in which expression of the *nrdEF* operon was modulated by isopropyl- β -D-thiogalactopyranoside (IPTG). Moreover, genetic inactivation of *ytcG*, predicted to encode a repressor (NrdR) of *nrdEF* in this strain, dramatically increased the expression levels of a transcriptional *nrdE-lacZ* fusion. The frequencies of mutations conferring amino acid prototrophy in three genes were measured in cultures under conditions that repressed or induced RNR-encoding genes. The results revealed that RNR was necessary for SPM and overexpression of *nrdEF* promoted growth-dependent mutagenesis and SPM. We also found that *nrdEF* expression was induced by H₂O₂ and such induction was dependent on the master regulator PerR. These observations strongly suggest that the metabolic conditions operating in starved *B. subtilis* cells increase the levels of RNR, which have a direct impact on SPM.

IMPORTANCE Results presented in this study support the concept that the adverse metabolic conditions prevailing in nutritionally stressed bacteria activate an oxidative stress response that disturbs ribonucleotide reductase (RNR) levels. Such an alteration of RNR levels promotes mutagenic events that allow *Bacillus subtilis* to escape from growth-limited conditions.

KEYWORDS *Bacillus subtilis*, stress-associated mutagenesis, ribonucleotide reductase

Ribonucleotide reductases (RNRs) are essential enzymes present in all life forms and are responsible for the conversion of ribonucleotides to 2'-deoxyribonucleotides, the precursors for the replication and repair of DNA. Three different RNR classes (classes I, II, and III) differing in the metal cofactor required for their catalytic activity, allosteric regulation, and quaternary structure have been described (1, 2). Class I RNRs are composed of two homodimeric proteins, α_2 and β_2 ; the larger subunit, α , contains the catalytic and the allosteric site that controls the specificity of reduction, while the smaller subunit, β , contains a stable tyrosyl radical and a dinuclear metal center (3, 4). In *Bacillus subtilis*, the *nrdE* and *nrdF* genes encode the α and β subunits of the class Ib RNR (3, 4). Although the activity of the RNRs is controlled mainly by allosteric regulation in *Escherichia coli* and other bacteria, the intracellular levels of this enzyme can also be regulated by different transcriptional factors, including DnaA, Fur, and NrdR. Such transcription factors respond to changes in metabolic or environmental conditions and mediate the repression or induction of RNR-encoding genes (2, 3, 5–7). The transcrip-

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Address correspondence to Mario Pedraza-Reyes, pedrama@ugto.mx.

tional repressor NrdR controls the expression of genes encoding the three RNR classes in virtually all bacteria studied, although it exerts a stronger effect over genes encoding class Ib proteins (7, 8).

The genetic alterations that occur in response to a persistent nonlethal selective pressure that allows organisms to escape from growth-limiting conditions are known as “adaptive mutagenesis” or “stationary-phase-associated mutagenesis” (SPM) (9–11). In *Bacillus subtilis*, strain YB955, bearing the chromosomal auxotrophies encoded by *hisC952*, *metB5*, and *leuC427*, has been successfully employed as a well-validated system to explore the mechanisms that promote adaptive mutations in soil bacteria (11). It has been suggested that under growth-limiting conditions, a subpopulation of cells in a culture may experience higher levels of DNA damage, activating error-prone repair events that promote adaptive mutagenesis (12–16). Several reports of studies with *B. subtilis* support this hypothesis; thus, *B. subtilis* cells lacking functional guanine-oxidized (GO) or mismatch repair (MMR) systems are highly prone to the accumulation of stationary-phase-associated mutations (17, 18). In nongrowing *B. subtilis* cells, saturation of the MMR system promotes mutagenesis through a mechanism that involves the glycosylase MutY and processing of A-C or A-G mispairs (15). Moreover, repair of deaminated cytosine in *B. subtilis* by the uracil DNA glycosylase Ung and the endonuclease V YwqL occurs in an error-prone manner, promoting adaptive mutagenesis (19). A recent report revealed that the numbers of spontaneously or enzymatically generated apurinic/apyrimidinic (AP) sites increase during the stationary phase in *B. subtilis* and that such lesions can be processed in an error-prone manner by the low-fidelity (LF) DNA polymerase PolX (20). Other reports have documented the participation of LF DNA polymerases, including YqjH (a member of the Y DNA polymerase family), in generating mutations in nutritionally stressed *B. subtilis* cells (21).

Compelling evidence has revealed that cellular levels of deoxynucleoside triphosphates (dNTPs) can affect the fidelity of replication. In growing *E. coli* cells, overexpression of RNR genes and the subsequent increase in dNTP pools not only stimulated the activity of translesion synthesis (TLS) DNA polymerases but also transiently modified the proofreading functions of replicative polymerases (22). A similar effect was observed in actively growing cells of *Saccharomyces cerevisiae*, where increased dNTP levels promoted mutagenic events by conferring on DNA polymerases the capacity to extend over DNA mismatches (23, 24). Therefore, it is clear that changes in RNR expression and subsequent dNTP levels affect mutagenesis in actively growing cells. However, little is currently known about how these processes impact the genetic changes occurring in nutritionally stressed nongrowing bacteria.

In the present work, the impact of RNR levels on growth-associated mutagenesis and SPM in *B. subtilis* YB955 (*hisC952 metB5 leuC427*) was investigated. Because the class Ib RNR encoded by the *nrdEF* genes is essential for growth (3, 4, 25), we used a conditional mutant to modulate *nrdEF* expression in growth-limited *B. subtilis* cells. Genetic disruption of *ycgG*, predicted to encode the NrdR repressor in *B. subtilis*, resulted in a strain that overexpressed the *nrdEF* operon. Analysis of *hisC952*, *metB5*, and *leuC427* reversion rates revealed that RNR-encoding genes are necessary for the production of adaptive His⁺, Met⁺, and Leu⁺ revertants. Notably, overexpression of *nrdEF* had a positive impact on the production of prototrophic His⁺, Met⁺, and Leu⁺ colonies during growth as well as on nutritionally stressed, nongrowing *B. subtilis* cells. Finally, we found that, in addition to the negative regulation exerted by NrdR, the expression of the *nrdEF* operon was affected by the oxidative stress response regulator PerR. In summary, our results support the concept that RNR levels are disturbed by the adverse metabolic conditions prevailing in nutritionally stressed cells and have a direct impact on *B. subtilis* SPM.

RESULTS

Construction and characterization of a conditional *nrdEF* mutant of *B. subtilis* YB955. *B. subtilis* YB955 (*hisC952 metB5 leuC427*) possesses a single essential class Ib

FIG 1 Diagram showing the recombination event that generated a conditional *nrdEF* mutant, *B. subtilis* PERM1017. Expression of *nrdE-nrdF* in this strain is under the control of the IPTG-inducible *Pspac* promoter. In this strain, a 5'-end fragment of *nrdE* is fused to *lacZ*, and the resulting *nrdE-lacZ* fusion and *nrdI* are under the transcriptional control of the native regulatory regions of the *nrdIEF* operon. The 3' end of the construct provides the cell with a functional RNR when IPTG is present; the 5' end of the construct indicates native promoter activity.

RNR whose subunits are encoded by the *nrdEF* operon (4); therefore, one would expect NrdEF to be the only protein responsible for generating 2'-deoxyribonucleotides for DNA replication and repair in this strain (3, 4). To investigate whether fluctuations in NrdEF levels impact mutagenesis in *B. subtilis* cells, we constructed a YB955 mutant in which the expression of *nrdEF* was conditioned by the presence of isopropyl- β -D-thiogalactopyranoside (IPTG) in the culture medium (Fig. 1). Of note, since the product of *nrdI*, the cistron preceding *nrdE-nrdF*, has been shown to be necessary for full RNR activity in other bacteria (26–28), expression of *nrdI* in the conditional *nrdE-nrdF* mutant was left under the control of the native regulatory regions of the *nrdI-nrdE-nrdF* operon (Fig. 1). Therefore, under inducing conditions, the conditional RNR mutant strain expresses *nrdE-nrdF* as well as *nrdI* for full RNR activity. As shown in Fig. 2A, as assessed by determination of the optical density at 600 nm (OD_{600}) and viable counts, the absence of IPTG in the culture medium abrogated the growth of this conditional mutant (PERM1017), whereas supplementation with $\geq 50 \mu\text{M}$ IPTG resulted in growth comparable to that of the parental strain in broth (Fig. 2B) or agar medium (Fig. 2C).

YtcG represses *nrdEF* expression in *B. subtilis*. It has been proposed that the *nrdEF* genes from *B. subtilis* are transcribed together with *nrdI* and *ymaB* as part of the *nrdIEF-ymaB* operon (3, 4). Further, results from a transcriptomic study revealed that *nrdE* and *nrdF* seem to be regulated by a DnaA-mediated transcriptional response (29, 30). Aside from this, little is known regarding additional factors involved in modulating *nrdEF* expression in *B. subtilis*. In this bacterium, the gene *ycgG* encodes a 152-amino-acid protein with 49% identity and 67% similarity to the transcriptional repressor NrdR from *E. coli* (7). Depending on the bacterial species, the length of the NrdR repressor varies from 150 to 200 amino acids. The N-terminal portion displays a zinc finger-like DNA-binding domain, whereas the C-terminal region encodes a putative class Ia ATP/dATP allosteric site (31–33). On the basis of these properties, it is proposed that increasing cellular levels of nucleotides activate the repressing properties of NrdR, which recognizes a palindromic element (NrdR box) located in the promoter region of the *nrdEF* operon (34, 35). In fact, results from an *in silico* analysis revealed the presence of two potential NrdR boxes in the regulatory region that control the expression of the *nrdIEF* operon (34). We then investigated whether YtcG is involved in regulating the expression of *nrdEF*; to this end, we first analyzed the expression patterns of *nrdEF* and *ycgG* during the life cycle of *B. subtilis*. *B. subtilis* strains bearing transcriptional in-frame *nrdEF-lacZ* and *ycgG-lacZ* fusions were propagated in antibiotic medium 3 (A3 medium [also called Penassay broth]; Difco Laboratories, Sparks, MD), and cell samples collected at different growth stages were assayed for β -galactosidase activity. As shown in Fig. 3A, expression of the *nrdEF-lacZ* fusion was maximal ($\sim 255 \pm 10$ Miller units) during exponential growth and decreased ~ 3 -fold during the transition and stationary phases of growth. On the other hand, the levels of β -galactosidase produced by the *B. subtilis* *ycgG-lacZ* strain were about 30-fold lower than those produced by the *nrdEF-lacZ* strain and remained barely constant during all the growth stages of *B. subtilis* (Fig. 3B). These results suggest that YtcG is present during the entire life cycle of *B. subtilis* putatively to modulate the levels of *nrdEF*. To determine whether *ycgG* encodes a functional repressor for *nrdEF* transcription, the level of β -galactosidase activity of the *nrdEF-lacZ*

FIG 2 (A) Transcriptional induction of *nrdEF* and growth phenotype of strain *B. subtilis* PERM1017 in the absence or presence of increasing amounts of IPTG. (B) Viable counts of cultures for which the results are shown in panel A during the exponential phase ($OD_{600} = 0.7$) and stationary phase ($OD_{600} = 1.6$) of growth. (C) Ability of *B. subtilis* strain PERM1017 to grow in minimal medium supplemented or not with increasing amounts of IPTG. *B. subtilis* PERM1017 was propagated in liquid LB supplemented with 1 mM IPTG until saturation. Cells collected by centrifugation were washed and resuspended in $1 \times$ SS. Serial dilutions of the cell suspension were plated in SMM supplemented or not with different concentrations of IPTG. Colony formation was monitored after 48 h of incubation at 37°C. 0, no IPTG addition; PS, parental strain YB955.

fusion strain was measured in a background with a defective *ytgG* gene. As shown in Fig. 3C, in reference to the YtcG-proficient strain, the level of *nrdE* expression was increased ~ 6 -fold during exponential, early, and late stages of growth in the YtcG-deficient strain. These results, together with the presence of two putative NrdR boxes upstream of *nrdIEF* overlapping -10 and -35 promoter elements (34), strongly suggest that (i) YtcG is the functional repressor for *nrdEF* transcription, (ii) its regulatory functions are exerted throughout the life cycle of *B. subtilis*, and (iii) the temporal pattern of *nrdEF* expression is independent of YtcG.

***nrdEF* expression in nutritionally stressed *B. subtilis* cells.** The strain *B. subtilis* YB955, bearing the chromosomal auxotrophic markers *hisC952*, *metB5*, and *leuC427*, has been successfully employed as a model system to understand how mutations are generated under conditions of limited growth (11, 15, 17–20, 36–39). It is possible that under those conditions dNTPs are required for DNA repair; however, the expression levels of *nrdEF* in *B. subtilis* cells under conditions of growth arrest are currently unknown. We investigated nondividing cells of strains YB955, PERM1017, and PERM1202 for the presence of *nrdE* transcript. These strains were grown and subsequently exposed to nutritional stress in agar medium lacking histidine, methionine, and leucine and incubated for a period of 8 days at 37°C. Reverse transcription-PCR (RT-PCR) analysis was performed with total RNA extracted from the three strains at the beginning of incubation and at 3 and 8 days of incubation. As shown in Fig. 4, in parental

FIG 3 Analysis of expression of *nrdE-lacZ* (A), *ytcG-lacZ* (B), and *ΔytcG nrdE-lacZ* (C) transcriptional fusions during exponential and stationary phases of *B. subtilis* growth. Strains were propagated in PAB medium. Cells collected at the indicated times were disrupted with lysozyme, and the cell extracts were assayed for β-galactosidase with ONPG as described in Materials and Methods. The data shown are average values from triplicate independent experiments ± standard deviations (SDs) for β-galactosidase specific activity (□) and for OD₆₀₀ (●).

strain YB955, the *nrdE* transcript levels decreased by about three times by day 8 of starvation. In contrast, in the conditional *nrdEF* mutant, in the absence of IPTG, the *nrdE* transcript levels decreased by about three times by day 4 and completely disappeared by day 8 of starvation (Fig. 4). On the other hand, the *ΔytcG* strain expressed about 1.8 times higher levels of the *nrdE* transcript than strain YB955 beginning on the first starvation day; moreover, such levels increased 3 and 6 times at days 3 and 8 poststarvation, respectively (Fig. 4).

Inactivation of *nrdEF* inhibits *B. subtilis* stationary-phase-associated mutagenesis. The conditional RNR mutant constructed in the YB955 genetic background (PERM1017) was used to establish the role of NrdEF in producing His⁺, Met⁺, and Leu⁺ adaptive revertants. To this end, after the mutant strain was propagated under permissive conditions, cells were collected at 90 min after the cessation of growth and

FIG 4 *nrdE* expression in starved *B. subtilis* cells. (A) RT-PCR analysis of *nrdE* expression in strains YB955, PERM1017 (*nrdEF*), and PERM1202 (*ytcG*) on day 1, 3, or 8 after incubation in 1× SMM lacking His, Met, Leu, and IPTG. (B) Densitometry analysis of the results shown in panel A using the *veg* gene as an endogenous control for constitutive expression (31). The results shown in panel B are average values from triplicate independent experiments ± SDs.

FIG 5 The frequencies of stationary-phase reversions to His⁺ (A), Met⁺ (B), and Leu⁺ (C) of *B. subtilis* strains YB955 (○), PERM1202 (Δ ytcG) (▲), and PERM1017 (*P*spac-*nrdEF*) (■) were determined as described in Materials and Methods. Data were normalized to the initial cell titers $\pm$ SDs and represent the counts averaged from three separate tests.

plated on Spizizen minimal medium (SMM) lacking IPTG and two of the required amino acids (histidine and leucine dropout medium or methionine and leucine dropout medium), and then after different periods of incubation, a set of plates was taken and supplemented with 0.1 mM IPTG (to induce *nrdEF* expression) and one of the initially absent amino acids. In this way, the initial absence of IPTG allowed us to temporarily inactivate *nrdEF* expression to evaluate the contribution of RNR to mutagenesis, whereas the subsequent addition of this agent guaranteed supplementation of the dNTPs required for DNA replication in the revertant cells. Moreover, the initial absence of two essential amino acids prevented the growth of revertants with variant or less efficient enzymes conferring growth (33, 36). Our results revealed that in comparison with the levels of reversion in parental strain YB955, inactivation of *nrdEF* expression in the conditional mutant PERM1017 induced a significant decrease in the levels of production of the His⁺, Met⁺, and Leu⁺ revertants (Fig. 5). Importantly, during the course of the experiments, it was shown that the absence of amino acids or IPTG did not affect the survival rates of the studied strains (see Fig. S1 in the supplemental material). Therefore, the levels of SPM-dependent His⁺, Met⁺, or Leu⁺ reversion obtained cannot be attributed to problems with the viability of the strains tested. Taken together, these results support the notion that NrdEF is required to provide the precursors necessary for events of DNA synthesis that promote mutations in nongrowing *B. subtilis* cells.

Overexpression of *nrdEF* promotes *B. subtilis* SPM. To establish the impact of RNR derepression in *B. subtilis* SPM, we determined the reversion frequencies to His⁺, Met⁺, and Leu⁺ in cultures of starved *B. subtilis* Δ ytcG cells. As shown in Fig. 5, the YtcG-deficient strain showed a significant increase in the frequencies of His⁺, Met⁺, and Leu⁺ reversions in reference to those generated by the strain proficient for this repressor. Importantly, as shown in Fig. S1, the levels of SPM-dependent amino acid reversions could not be attributed to differences in the viabilities of the strains. Together, these results and those from the experiments in which *nrdEF* was repressed strongly support the participation of RNR in *B. subtilis* SPM.

Analysis of suppressor mutations in the *nrdEF*-overexpressing strain. Histidine and methionine auxotrophies were generated by nonsense mutations in the *hisC952* and *metB5* genes, and then it was suggested that His⁺ Met⁺ revertants could be generated by a suppressor *sup-3* allele that simultaneously confers prototrophy to both His⁺ and Met⁺ revertants, in addition to other less well characterized suppressors (11, 40). Our analysis for suppressor mutations showed that in strain YB955 a high percentage (47%) of the Met⁺ revertants presented a His⁺ phenotype; moreover, 10% of the His⁺ colonies were also Met⁺ revertants (Table 1), suggesting that the His⁺ Met⁺ prototrophs were also generated by suppressor mutations. As also shown in Table 1, repression of *nrdEF* in the conditional mutant did not promote a significant change in the proportion of His⁺ Met⁺ colonies in comparison with that for YB955. Of note, the

TABLE 1 Growth of stationary-phase revertants on alternative selection media^d

Strain and selection medium	No. of revertants that showed the following phenotype/no. of revertants analyzed (%)				
	His ⁺ Met ⁺ Leu ⁺	His ⁺ Met ⁺	Met ⁺ Leu ⁺	His ⁺ Leu ⁺	Single revertant (His ⁺ , Met ⁺ , or Leu ⁺)
<i>ΔytcG</i> strain					
His [−]	27/100 (27)			2/100 (2)	71/100 (71) ^a
Met [−]	7/100 (7)	4/100 (4)			89/100 (89) ^b
Leu [−]	22/100 (22)		7/100 (7)	2/100 (2)	69/100 (69) ^c
YB955					
His [−]	3/100 (3)	10/100 (10)			87/100 (77) ^a
Met [−]	2/100 (2)	47/100 (47)			51/100 (51) ^b
Leu [−]					100/100 (100) ^c
<i>Pspac-nrdEF</i> strain (with <i>nrdEF</i> inactivated)					
His [−]		3/54 (5)			51/54 (95) ^a
Met [−]		5/19 (26)			14/19 (74) ^b
Leu [−]					50/50

^aHis⁺ revertants.^bMet⁺ revertants.^cLeu⁺ revertants.^dHis⁺, Met⁺, and Leu⁺ revertants from day 4, 6, or 8 were screened on 1× SMM missing one required amino acid (His, Met, or Leu [His[−], Met[−], or Leu[−], respectively]) to screen for suppressor mutations. Plates were scored after 48 h of incubation.

absence of YtcG (NrdR) generated a lower but more diverse group of suppressors bearing the His⁺ Met⁺ (4%), Leu⁺ Met⁺ (7%), His⁺ Leu⁺ (2%), and Leu⁺ His⁺ (2%) phenotypes.

Derepression of *nrdEF* promotes the formation of intragenic suppressors. The high propensity to accumulate adaptive colonies with reversion in the three genes tested exhibited by the YtcG-deficient strain prompted us to investigate the type of mutation that gave rise to this phenotype. The *hisC952*, *metB5*, and *leuC427* genes of 30 independent adaptive His⁺ Met⁺ Leu⁺ revertants generated at day 3, 4, or 5 by the YtcG-deficient strain were sequenced. Surprisingly, about two-thirds of the colonies analyzed contained changes that restored the wild-type sequence of the three genes analyzed (Table 2). However, the genetic changes associated with one-third of the triple His⁺ Met⁺ Leu⁺ revertants were not identified in the amplicons sequenced, suggesting that missense suppressors generated these types of revertants (Table 2). Sequencing analysis of single Met⁺, His⁺, or Leu⁺ revertants revealed the presence of mutations that changed the stop codons to distinct amino acid codons in the first two types of revertants. The mutations that reestablished the Leu⁺ phenotype were not located in the mutant codon of the *leuC427* gene.

Mutation rates in growing *B. subtilis* cells with derepressed *nrdEF*. The reversion levels of the *hisC952*, *metB5*, and *leuC427* alleles in exponentially growing cells of the strain deficient for the repressor YtcG were determined and compared with those generated in parental strain YB955. As shown in Fig. S2, the levels of production of His⁺, Met⁺, or Leu⁺ revertants by the *ΔytcG* strain increased ~2.5, 2, and 1.5 times, respectively, compared to those by the YB955 parental strain. Therefore, higher levels of RNR also promoted mutagenesis in growing *B. subtilis* cells.

Transcription of *nrdEF-lacZ* is increased by oxidative stress. In *E. coli*, *Staphylococcus aureus*, *Bacillus anthracis*, and *Corynebacterium ammoniagenes*, genes coding for class Ib RNR are induced in response to oxidative stress (41–45); therefore, we inquired whether *B. subtilis nrdEF* could be also induced by the reactive oxygen species-promoting agent hydrogen peroxide (41, 44, 46–48). To investigate this point, strain PERM1017 carrying the *nrdE-lacZ* fusion was cultured in A3 medium to an OD₆₀₀ of 0.5 and treated with hydrogen peroxide (1 mM). The results revealed that exposure to H₂O₂ increased the level of *nrdEF* expression 1.5-fold compared to that in a nontreated culture (Fig. 6A). In *B. subtilis*, the PerR repressor controls the expression of an oxidative stress regulon that is triggered by the presence of H₂O₂ in the culture medium (46, 48).

TABLE 2 Base changes in revertants of mutant alleles^e

Phenotype and revertant allele	Position(s) of mutation (bp)	No. of revertants with mutation/no. of revertants sequenced	Type of mutation	DNA change	Result of mutation
His⁺ Met⁺ Leu⁺a					
<i>hisC</i>	952	22/30	Transition	T → C	Stop → Gln
<i>hisC</i>	NF	8/30	NF	NF	NF
<i>metB</i>	346	19/30	Transversion	T → G	Stop → Glu
<i>metB</i>	346 and 348	2/30	Transversion	T → G and A → T	Stop → Asp
<i>metB</i>	NF	9/30	NF	NF	NF
<i>leuC</i>	427	19/30	Transition	A → G	Arg → Gly
<i>leuC</i>	NF	11/30	NF	NF	NF
Met⁺b					
<i>metB</i>	346	6/10	Transition	T → C	Stop → Gln
<i>metB</i>	346	2/10	Transition	T → A	Stop → Lys
<i>metB</i>	NF	2/10	NF	NF	NF
His⁺c					
<i>hisC</i>	952	4/10	Transition	T → C	Stop → Gln
<i>hisC</i>	952	2/10	Transversion	T → G	Stop → Glu
<i>hisC</i>	953	4/10	Transition	A → G	Stop → Trp
Leu⁺,^d <i>leuC</i>	NF	10/10	NF	NF	NF

^aRevertants were selected in histidine dropout medium (7/30 revertants) or methionine dropout medium (23/30 revertants).

^bRevertants were selected in methionine dropout medium (10 revertants).

^cRevertants were selected in histidine dropout medium (10 revertants).

^dRevertants were selected in leucine dropout medium (10 revertants).

^eRevertants were tested for their ability to grow on 1× SMM lacking histidine, methionine, and leucine and classified for their phenotype. Fragments of the *hisC*, *metB*, and *leuC* genes were sequenced depending on the observed reversion. NF, no changes were found in the sequenced fragment.

As shown in Fig. 6B, disruption of *perR* increased about five times the expression levels of the *nrdE-lacZ* fusion in comparison with the level for the *B. subtilis* strain that was proficient for PerR. A similar result was found for group A *Streptococcus*, where expression of the ribonucleotide reductase *nrdF2-nrdI2-nrdE2* operon was directly regulated by the PerR repressor (47). Altogether, our results suggest that in *B. subtilis*, PerR directly or indirectly regulates the expression of RNR-encoding genes in response to oxidative stress.

FIG 6 H_2O_2 induction of an *nrdE-lacZ* transcriptional fusion and its expression in *B. subtilis* strains deficient or not for PerR. (A) Induction of *nrdE-lacZ* fusion in *B. subtilis* after exposure to H_2O_2 . *B. subtilis* PERM1017 was propagated in A3 medium to an OD_{600} of 0.5. At this point the culture was equally divided into two flasks. One of the subcultures was left untreated (circles), and the other one was challenged with 1 mM H_2O_2 (triangles). Cells harvested at the indicated times were assayed for β -galactosidase activity as described in Materials and Methods. Values represent averages from triplicate independent experiments $\pm$ SDs. (B) Expression of an *nrdE-lacZ* fusion in a PerR genetic background. The levels of β -galactosidase produced by *B. subtilis* strains PERM1017 (*nrdE-lacZ*) (circles) and PERM1159 (*perR nrdE-lacZ*) (triangles) are shown. Strains were grown in A3 medium. Cell samples were collected at the indicated times and treated with lysozyme, and the extracts were assayed for β -galactosidase as described in Materials and Methods. The data shown are average values from triplicate independent experiments $\pm$ SDs.

DISCUSSION

Ribonucleotide reductases, the rate-limiting factor for the synthesis of 2'-deoxyribonucleotides, are subjected to extensive allosteric regulation in most organisms (1, 2). However, the intracellular amount of these enzymes is also regulated at the transcriptional level; such control is of particular relevance in bacterial species possessing different types of RNRs, including *E. coli*, *Salmonella*, and *Pseudomonas aeruginosa* (7, 8, 35). *B. subtilis* possesses one class Ib RNR, encoded by the *nrdEF* operon (3, 4). Disruption of either the *nrdE* or the *nrdF* open reading frame generates a growth arrest in this microorganism (4). Studies aimed at understanding the physiological role of NrdEF in *B. subtilis* have been performed with temperature-sensitive conditional mutants. These studies have employed 45°C as the nonpermissive condition (25). However, under these conditions the heat shock regulon is activated (49, 50) and may confound the effects of RNR activity. The conditional mutant *B. subtilis* YB955 *Pspac-nrdEF*, constructed in this work, permitted analysis of the mutagenic events leading to the production of colonies with His⁺, Met⁺, and Leu⁺ phenotypes under growth-limiting conditions and at 37°C. This avoided the undesired pleiotropic or confounding effects triggered at 45°C. Importantly, the amount of IPTG (namely, 0.1 mM) chosen to propagate the conditional mutant promoted growth levels indistinguishable from those of the parental strain (Fig. 2). Furthermore, experiments employing a different marker for mutagenesis (i.e., the *rpoB* gene) demonstrated that increased levels of *nrdEF* expression correlated with increased frequencies of mutation to rifampin resistance (Rif^r) in the conditional mutant strain PERM1017 (see Fig. S3 in the supplemental material).

In *B. subtilis*, the DNA region preceding the *nrdIEF* operon possesses two putative conserved DNA sequences predicted to be bound by NrdR (31). This protein represses the expression of genes encoding the class 1b RNR in *E. coli* and other bacteria (7, 8, 35). *B. subtilis* contains a putative ortholog of this protein encoded by *ytcG*. In this work, we showed that disruption of this gene resulted in the increased expression of an *nrdE-lacZ* transcriptional fusion, strongly suggesting that YtcG corresponds to the NrdR ortholog in this microorganism.

Oxidative stress is an important factor that promotes adaptive mutagenesis in *B. subtilis* (18); thus, deficiency of the GO system exacerbated the production of His⁺ and Met⁺ revertants in nondividing cells of this microorganism (18). Interestingly, as previously described for group A *Streptococcus* (47), our results showed that the oxidative stress promoted by H₂O₂ activated the expression of the *nrdEF* operon. Further, *nrdEF* expression was significantly increased in a *B. subtilis* genetic background deficient for the repressor PerR. Of note, conditions that interfere with replication or cell elongation in this microorganism induce PerR as well as the RNR operon in a way dependent on DnaA (30). On the basis of these results, it is feasible to propose that the adverse metabolic conditions operating in nutritionally stressed cells may affect RNR levels. Our results also showed that repression of *nrdEF* transcription in strain PERM1017 induced a significant decrease in the levels of production of adaptive His⁺, Met⁺, and Leu⁺ revertants compared to those for the parental strain. In contrast, the RNR conditional mutant deficient in YtcG overexpressed the *nrdEF* operon and displayed increases, by severalfold, in reversion in the three genes tested (Fig. 5). These results revealed that reversions in the tested genes increased in parallel with *nrdEF* induction. Interestingly, the expression levels of *nrdE* decreased in the nonrevertant populations of YB955 and PERM1017 under conditions of prolonged starvation; however, a fraction of starved YB955 cells produced revertants (Fig. 5), suggesting that changes in RNR transcription take place in a subpopulation of cells to promote mutagenic events. Previous reports revealed that adaptive mutations arise in a subpopulation of nutritionally stressed nongrowing, bacterial cells (11, 17). Taken together, our results support the concept that induction of RNR is necessary to generate adaptive mutations in *B. subtilis* and suggest that the metabolic conditions prevailing in nongrowing cells

increase the expression levels of RNR, which have a direct impact on stationary-phase-associated mutagenesis (SPM).

Our suppressor analysis revealed that an important proportion of the His⁺ and Met⁺ colonies generated by the conditional *nrdeF* mutant were also prototrophic for methionine or leucine, respectively, whereas none of the Leu⁺ colonies tested exhibited additional amino acid prototrophies (Table 1). Of note, these analyses also revealed that a very high proportion (~20%) of the His⁺, Met⁺, or Leu⁺ revertants generated by nondividing cells of the strain deficient for NrdR acquired mutations that conferred prototrophy in two other genes (Table 1). Remarkably, DNA sequencing analysis showed that about two-thirds of the His⁺ Met⁺ Leu⁺ colonies carried mutations that changed not only the missense *leuC427* but also the nonsense *hisC952* and *metB5* genes to amino acid codons that reestablished the wild-type sequences of their open reading frames. Moreover, in one-third of the triple His⁺ Met⁺ Leu⁺ revertants, the mutations were not detected in the defective genes; however, these colonies exhibited robust growth in each of the three selective media (histidine dropout medium, methionine dropout medium, and leucine dropout medium). Further sequence analyses showed differences between mutant colonies bearing triple and single reversions; for instance, Met⁺ reversions in the triple His⁺ Met⁺ Leu⁺ colonies mainly resulted from a T → G transversion event. In contrast, T → C and T → A suppressor mutations were involved in generating colonies with single Met⁺ reversions. Taken together, these results point to the existence of a common mechanism that increases the occurrence of mutations in genes under selection in starved cells of *B. subtilis* deprived of NrdR.

In starved *B. subtilis* YB955 cells, true reversions to His⁺, Met⁺, and Leu⁺ have been associated with transcription repair coupling events mediated by Mfd (38); notably, the frequency of Mfd-dependent true reversions increased proportionally to the transcription levels of the gene under selection (36, 37). Perhaps the high proportion of true reversions in starved cells overexpressing the RNR-encoding genes is linked to mutagenic events occurring at genes undergoing transcriptional derepression. Since our assays were conducted in the absence of histidine, methionine, or leucine, one would expect that genes like *hisC955*, *metB5*, and *leuC427* would be highly transcribed and would be subjected to transcription-mediated mutagenesis.

The stringent response, activated during starvation and mediated by GTP levels, induces the expression of genes involved in amino acid biosynthesis (51). Such a response has been shown to increase bacterial survival during amino acid starvation (52). In contrast, high concentrations of GTP activate CodY, which represses the expression of genes required for the synthesis and utilization of branched-chain amino acids (BCAAs), in addition to methionine, histidine, and arginine, among others (52, 53). Furthermore, it has been reported that changes in RNR levels affect the fidelity of DNA synthesis by increasing and/or biasing the dNTP pools (22–24, 54, 55). In agreement with these observations, in nongrowing *B. subtilis* cells, the low-fidelity DNA polymerases PolX, YqjH, and/or PolA have been shown to promote error-prone repair events of DNA synthesis (20, 21, 56–59).

Based on these observations, we propose that in growth-arrested *B. subtilis* cells, changes in expression of RNR genes alters dNTP/NTP pools. Changes in dNTP/NTP pools precipitate the activation of the stringent response; change transcription, particularly in amino acid biosynthetic genes; and alter the fidelity of DNA polymerases. All these conditions lead to a mutagenic program that increases the likelihood that stressed cells will escape from growth-limiting conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains and plasmids used in this study are listed in Table 3. The procedures used for the transformation and isolation of chromosomal and plasmid DNA were described previously (60, 61). Liquid cultures of *B. subtilis* strains were routinely grown in Penassay broth (PAB; Difco Laboratories, Sparks, MD). Growth was monitored with a Pharmacia Ultrospec 2000 spectrophotometer set at 600 nm. When required, erythromycin (Ery; 1 μg/ml), neomycin (Neo; 10 μg/ml), kanamycin (Kan; 10 μg/ml), or isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) was added to

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Genotype or description ^a	Reference, source, or transformation ^b
Strains		
<i>B. subtilis</i>		
YB955	<i>hisC952 metB5 leuC427 xin-1 Spβ^s</i>	11
HB2078	CU1065 <i>ΔperR::kan Kan^r</i>	46
PERM1017	<i>ΔnrdE::lacZ Pspac-nrdEF::ery Ery^r</i>	pPERM1011 → YB955
PERM1202	<i>ΔytcG::neo Neo^r</i>	pPERM1179 → YB955
PERM1207	<i>ΔytcG::neo ΔnrdE::lacZ Pspac-nrdEF::ery Neo^r Ery^r</i>	pPERM1179 → PERM1017
PERM1159	<i>ΔperR::kan ΔnrdE::lacZ Pspac-nrdEF::ery Kan^r Ery^r</i>	pPERM1011 → HB2078
PERM1448	<i>ytcG::lacZ</i>	pPERM1330 → YB955
<i>E. coli</i> XL10-Gold		
	<i>endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)Tn10 Tet^r Cm^r</i>	Laboratory stock
Plasmids		
pMUTIN4	Integrational <i>lacZ</i> fusion vector; Amp ^r Ery ^r	62
pBEST501	Integrational neomycin replacement vector; Amp ^r Neo ^r	63
pPERM1011	pMUTIN4 containing a 5' fragment (from bp −47 to 290) of the <i>nrdE</i> ORF cloned into the HindIII-SacII sites; Amp ^r Ery ^r	This study
pPERM1179	pBEST501 containing a 5' fragment (from bp −367 to −22) and a 3' fragment (from bp +23 to +365) adjacent to the <i>ytcG</i> ORF cloned into the HindIII-SacI and BamHI-SacI sites, respectively; Amp ^r Ery ^r	This study
pPERM1330	pMUTIN4 containing a 3' fragment (from bp −179 to 456) of the <i>ytcG</i> ORF cloned into BamHI-EcoRI sites; Amp ^r Ery ^r	This study

^aAmp, ampicillin; Ery, erythromycin; Neo, neomycin; Kan, kanamycin; ORF, open reading frame.

^bTransformations are represented by X → Y, where strain Y was transformed with plasmid DNA from source X.

the medium. PCR products were obtained with homologous oligonucleotide primers (Table 4) and Vent DNA polymerase (New England Biolabs, Ipswich, MA).

Construction of mutant strains. To obtain a conditional *nrdEF* mutant in the *B. subtilis* YB955 genetic background, a 333-bp HindIII-SacII 5' fragment of the *nrdE* gene (from bp −47 to bp 290 of the *nrdE* translational start codon) was amplified by PCR using chromosomal DNA from *B. subtilis* 168 and oligonucleotide primers 1 and 2 (Table 4). The PCR fragment was inserted between the HindIII and SacII sites of the integrative plasmid pMUTIN4 (62). The resulting construction, designated pPERM1011 (*nrdE::lacZ*), was used to transform *B. subtilis* YB955, generating strain PERM1017 (*Pspac-nrdEF*) (Fig. 1).

To generate a *ytcG* (*nrdR*) mutant, a 345-bp fragment from the 5' *ytcG* region (nucleotides [nt] −367 to −22 relative to the *ytcG* start codon) and a 342-bp fragment from the 3' *ytcG* region (nt +23 to +365 relative to the *ytcG* stop codon) were PCR amplified using chromosomal DNA from *B. subtilis* 168. Oligonucleotide primers 3 and 4 and oligonucleotide primers 5 and 6 (Table 4) were used for amplification of the 5' *ytcG* and 3' *ytcG* fragments, respectively. The amplified 5' *ytcG* and 3' *ytcG* fragments were cloned between the HindIII/SacI and BamHI/SacI sites of pBEST501 (63), respectively. The resulting construction, designated pPERM1179, was propagated in *E. coli* XL10-Gold cells and used to transform competent cells of *B. subtilis* YB955 to generate strain PERM1202 (*ΔytcG*). The double-crossover event replacing *ytcG* with a neomycin resistance cassette was confirmed by PCR (results not shown).

Construction of *ytcG-lacZ* and *nrdE-lacZ* fusions. To generate a strain carrying a *ytcG-lacZ* fusion, a fragment from positions 179 to 456 of the *ytcG* open reading frame was amplified by PCR using chromosomal DNA from *B. subtilis* 168 and oligonucleotide primers 7 and 8 (Table 4). The PCR product was cloned between the BamHI and EcoRI sites of the integrative plasmid pMUTIN4 immediately upstream of the *lacZ* gene. The resulting construct, designated pPERM1330, was used to transform *B. subtilis* YB955, generating strain PERM1448 carrying a transcriptional in-frame *ytcG-lacZ* fusion.

To recombine the transcriptional in-frame *nrdE-lacZ* fusion in a *PerR*-deficient genetic background, competent cells of strain *B. subtilis* HB2078 (*perR*) (46) were transformed with plasmid pPERM1011, thus generating strain *B. subtilis* PERM1159 (Table 3). The correct integration of the construction into the *nrdE* locus was verified by PCR using specific oligonucleotide primers (data not shown).

Stationary-phase mutagenesis soft-agar overlay assays. The stationary-phase mutagenesis assays were performed as previously described (36) with modifications. Strains of *B. subtilis* were grown in 10 ml of PAB medium to 90 min after time zero, which was the time point in the culture when the slopes of the logarithmic and stationary phases of growth intercepted. After centrifugation, the cell pellets were washed 3 times and resuspended in 10 ml of 1× Spizizen minimal salts (1× SS) (64). Aliquots of 100 μl were plated onto Spizizen minimal medium (SMM; 1.5% agar, 1× SS, 0.5% glucose, 50 μg/ml isoleucine, 50 μg/ml glutamic acid; the content of histidine, methionine, and leucine depended on the reversion assay). Two types of minimal medium were used: histidine and leucine dropout medium (SMM with 50 μg/ml of methionine, 200 ng/ml of leucine, and 200 ng/ml of histidine) and methionine and leucine dropout medium (SMM with 50 μg/ml of histidine, 200 ng/ml of leucine, and 200 ng/ml of methionine). Starting from 48 h and every 2 days thereafter, a set of plates was overlaid with soft agar (0.7% agar

TABLE 4 Oligonucleotides used for PCRs

Primer no.	Oligonucleotide ^a	Sequence ^b (5' to 3')	Amplified region
1	F- <i>nrdE</i>	GAAGCTTCGGCACATCTAAAGA	nt -47 to 290 relative to translational start codon of <i>nrdE</i>
2	R- <i>nrdE</i>	CGCCGCGGCACTCATGAAGGAAG	
3	F- <i>ytG</i> -US	CGCCGCGGCACTCATGAAGGAAG	nt -367 to -22 relative to translational start codon of <i>ytG</i>
4	R- <i>ytG</i> -US	CGGTCGACCCCAACTATATCGTTCCCG	
5	F- <i>ytG</i> -DS	GCGGATCCGCTAAGAGGATTCTTAGC	nt +23 to +365 relative to translational stop codon of <i>ytG</i>
6	R- <i>ytG</i> -DS	GCGAGCTCCCTGTTTCTGATGGATCGT	
7	F- <i>ytG</i>	GGATCCAAATGCTTTCATGCCA	nt 179 to 456 of <i>ytG</i> open reading frame
8	R- <i>ytG</i>	GAATTCCTCTTTCTTTATTAAGTC	
9	F- <i>hisC</i>	GCAGGCCTTCAGCAGTATTATGAT	nt 840 to +71 relative to translational stop codon of <i>hisC</i>
10	R- <i>hisC</i>	GACCGCGGAGCAATATTGTATCTTTCA	
11	F- <i>metB</i>	ATCCCAAGCACACACACGCCG	nt 210 to 516 of <i>metB</i> open reading frame
12	R- <i>metB</i>	AACGGTATGTTTGAACACACCGAAGAT	
13	F- <i>leuC</i>	CGCTTCCAGGAAAAACGATTG	nt 353 to 655 of <i>leuC</i> open reading frame
14	R- <i>leuC</i>	CGATGGATGAACGAATGACTG	
15	F- <i>nrdE</i>	GCTGCAGGCATCTCAAGTTT	nt 1155 to 1344 of <i>nrdE</i> open reading frame
16	R- <i>nrdE</i>	CGCATTACGGATGTCTGTTG	
17	F- <i>veg</i>	TGGCGAAGACGTTGTCCGATATTA	nt 2 to 83 of <i>veg</i> open reading frame
18	R- <i>veg</i>	CGGCCACCGTTTGCTTTTAAC	

^aF, forward; R, reverse.^bUnderlining indicates the following restriction sites: primers 1 and 3, HindIII; primer 2, SacII; primer 4, Sall; primers 5 and 7, BamHI; primer 6, SacI; and primer 8, EcoRI.

prewarmed at 42°C) amended with one of the amino acids initially absent to select His⁺, Met⁺, or Leu⁺ revertants. For strain PERM1017, the soft agar was supplemented with IPTG to a final concentration of 1 mM to induce expression of the *nrdE* genes. The plates were incubated for 2 days, and the number of revertant colonies was scored. The initial number of bacteria plated for each experiment was determined by serial dilution of the bacterial cultures that were plated on SMM containing all three essential amino acids. The experiments were repeated at least three times.

Cell survival was determined by removing three agar plugs daily, using sterile Pasteur pipettes, from areas of the plates where no growth of revertants was observed (20). The plugs were suspended in 400 μ l of 1 $\times$ SS, mixed, diluted, and plated on SMM containing all the essential amino acids (50 μ g/ml). The number of colonies was determined following 48 h of incubation at 37°C.

Fluctuation test. The growth-dependent reversion rates for His, Met, and Leu were measured by fluctuation tests with the Lea-Coulson formula (65, 66), as previously described (11, 15, 18, 20).

Analysis of frequencies of mutation to Rif^r. The frequencies of spontaneous mutation to rifampin resistance (Rif^r) were determined as previously described (18). Essentially, the appropriate strains were grown for 12 h at 37°C in antibiotic A3 medium with proper antibiotics. Independent cultures of strain PERM1017 were amended with 0.025, 0.1, or 1 mM IPTG, whereas the Δ *nrdR* strain was cultured in the absence of the inducer. Mutation frequencies were determined by plating aliquots on six LB plates containing 10 μ g ml⁻¹ rifampin, and the 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 plates without rifampin and incubating the plates for 24 to 48 h at 37°C. These experiments were repeated at least three times.

β -Galactosidase assays. *B. subtilis* strains PERM1017 and PERM1207, both of which contain the transcriptional *nrdE-lacZ* fusion, were propagated in liquid A3 medium supplemented with 0.1 mM IPTG. Aliquots of 0.5 ml were collected from the cultures during the exponential and stationary phases of growth at the times indicated in Fig. 3 and 6 and processed for determination of β -galactosidase activity using *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate (67).

DNA sequencing. Revertant colonies with the Δ *ytG* background were collected from plates from the stationary-phase mutagenesis assays on days 3 to 5 of incubation, independently propagated in liquid A3 medium, and subjected to DNA isolation. Internal fragments of the genes *hisC952*, *metB5*, and *leuC427* were amplified by PCR using high-fidelity DNA polymerase and specific oligonucleotide primers (Table 4). Sequencing services were carried out by Functional Biosciences, Inc. (Madison, WI).

RT-PCR. Strains YB955, PERM1017, and PERM1202 were grown under the conditions of the stationary-phase mutagenesis assays described above. Post-exponential-phase cultures (10 ml) were centrifuged, and the cell pellets were resuspended in 1 ml of 1 $\times$ SS. Aliquots (100 μ l) of this suspension were plated onto SMM containing growth-limiting amounts of histidine, methionine, and leucine and incubated at 37°C. At different intervals of incubation, the nonrevertant background was scraped off the agar medium with 2 ml of 1 $\times$ SS and recovered by centrifugation. Total RNA was isolated by using a Tri

Reagent kit (Molecular Research Center, Inc. Cincinnati, OH). Reverse transcription-PCRs (RT-PCRs) were performed with RNA samples and the Verso reverse transcriptase enzyme from a 1-step quantitative RT-PCR kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Primers 15 and 16 (Table 4) were used to amplify an 81-bp RT-PCR product extending from nt 2 to 83 of the *veg* transcript, which was used as an internal control for constitutive expression (36), whereas primers 17 and 18 (Table 4) generated a 189-bp RT-PCR product extending from positions 1155 to 1344 of the *nrdE* transcript. 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 products was determined by utilizing a 1-kb-Plus DNA ladder (Life Technologies, Rockville, MD) during agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed using a Gel Doc EZ imager (Bio-Rad Laboratories, Hercules CA); digital photographic images of the gels were scanned, and the RT-PCR products were quantified by densitometry using Image Lab (version 4.1) software and the *veg* RT-PCR product corresponding to each condition as a reference.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00715-16>.

TEXT S1, PDF file, 0.2 MB.

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