JOURNAL OF BACTERIOLOGY, Aug. 2010, p. 3915–3924 Vol. 192, No. 15
0021-9193/10/$12.00 doi:10.1128/JB.00210-10

Changes in DnaA-Dependent Gene Expression Contribute to the Transcriptional and Developmental Response of *Bacillus subtilis* to Manganese Limitation in Luria-Bertani Medium▽†

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Received 26 February 2010/Accepted 19 May 2010

The SOS response to DNA damage in bacteria is a well-known component of the complex transcriptional responses to genotoxic environmental stresses such as exposure to reactive oxygen species, alkylating agents, and many of the antibiotics targeting DNA replication. However, bacteria such as *Bacillus subtilis* also respond to conditions that perturb DNA replication via a transcriptional response mediated by the replication initiation protein DnaA. In addition to regulating the initiation of DNA replication, DnaA directly regulates the transcription of specific genes. Conditions that perturb DNA replication can trigger the accumulation of active DnaA, activating or repressing the transcription of genes in the DnaA regulon. We report here that simply growing *B. subtilis* in LB medium altered DnaA-dependent gene expression in a manner consistent with the accumulation of active DnaA and that this was part of a general transcriptional response to manganese limitation. The SOS response to DNA damage was not induced under these conditions. One of the genes positively regulated by DnaA in *Bacillus subtilis* encodes a protein that inhibits the initiation of sporulation, Sda. Sda expression was induced as cells entered stationary phase in LB medium but not in LB medium supplemented with manganese, and the induction of Sda inhibited sporulation-specific gene expression and the onset of spore morphogenesis. In the absence of Sda, manganese-limited cells initiated spore development but failed to form mature spores. These data highlight that DnaA-dependent gene expression may influence the response of bacteria to a range of environmental conditions, including conditions that are not obviously associated with genotoxic stress.

Several species of bacteria, including *Bacillus subtilis* and *Escherichia coli*, respond to conditions that perturb DNA replication through at least two independent signaling pathways: the well-known SOS response to DNA damage and an increasingly well-characterized signaling pathway mediated by the replication initiation factor DnaA. DnaA is a site-specific DNA binding protein that recognizes the origin of replication and initiates the assembly of the DNA replication machinery (32, 42). DnaA also functions as a transcription factor, recognizing specific promoters and activating or repressing the transcription of target genes (7, 25, 30, 36, 37, 54). The activity of DnaA is regulated in response to a variety of cell cycle-dependent, developmental, and nutritional signals, only some of which are understood at a mechanistic level for specific organisms (for recent reviews, see references 14, 16, 33, 36, 56, and 57). However, it is clear that some conditions that perturb DNA replication result in the accumulation of active DnaA and the induction or repression of DnaA-dependent gene expression (7, 20, 25, 35). Based on a limited number of studies, some conditions that induce the SOS response also alter DnaA-dependent gene expression, whereas other perturbations affect only one signaling pathway or the other (20, 21).

The SOS response to DNA damage clearly contributes to the transcriptional and adaptive responses of bacteria to environmental stresses such as reactive oxygen species and other DNA-damaging agents. Far less is known about when and how DnaA-dependent gene expression might affect the transcriptional or adaptive responses of bacteria to stressful or changing environments. Here we report that the growth of *B. subtilis* in LB medium alters DnaA-dependent gene expression as part of a general response to manganese limitation.

The effects of manganese on gene expression have been well characterized for *B. subtilis* (11, 17, 22, 24). Manganese is transported into cells by at least two different transport systems encoded by the *mntH* and *mntABCD* genes (48). The transcription of these genes is repressed at high manganese concentrations by MntR, which binds manganese as a corepressor (19, 22, 48). Manganese also represses the transcription of genes in the PerR and Fur regulons, which encode oxidative stress response proteins and iron acquisition proteins, respectively (11, 17, 22, 24). Although this is likely due in part to the roles of Mn(II) in scavenging reactive oxygen species and promoting the activities of SodA and other manganese-dependent enzymes, Mn(II) also affects PerR-dependent gene expression directly by replacing Fe(II) as a corepressor bound to PerR (24). The PerR-Mn(II) complex is much more resistant to inactivation by H_2O_2 than is the PerR-Fe(II) complex, limiting the expression of PerR-regulated genes (17, 24).

We realized that manganese limitation might also alter the expression of DnaA-dependent genes after we observed that a gene positively regulated by DnaA, *sda*, was induced when cells

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 28 May 2010.

were grown on LB medium but not on LB medium supplemented with manganese. The *sda* gene encodes an inhibitor of sporulation, a developmental response of *B. subtilis* to nutrient limitation at a high cell density (9, 50). Cells of *B. subtilis* initiate spore development with a specialized cell cycle, in which cells switch from dividing at the midcell and instead divide at one pole to create a large cell, termed the mother cell, and a small cell, termed the forespore, each of which inherits a single copy of the chromosome (16, 26). The initiation of sporulation is coordinated with the successful completion of DNA replication in part by the transcriptional regulation of *sda* (9, 51, 54). The transcription of *sda* is convergently regulated by DnaA and the SOS response, and *sda* expression is induced as cells enter the stationary phase if DNA replication initiation or elongation is perturbed and in response to DNA damage (7, 9, 20, 21, 30, 43, 51, 54). In addition, the transcription of *sda* is transiently induced by DnaA in the absence of replication stress as cells enter the stationary phase, delaying the initiation of sporulation until ongoing rounds of DNA replication are completed (54).

We show here that manganese limitation in LB medium induces *sda* transcription and alters the expression of other DnaA-dependent genes without inducing the SOS response. Thus, environmental conditions that are not obviously associated with genotoxic stress can affect DnaA-dependent gene expression, perhaps reflecting direct or indirect effects on DNA replication. In addition, DnaA-dependent changes in gene expression can contribute significantly to the transcriptional and adaptive responses of bacteria to stressful or changing environmental conditions.

MATERIALS AND METHODS

Media and strains. Strains were grown in LB medium (38) supplemented with 100 μM $MnCl_2$ where noted. T-base medium (1× TSS salts [2 g liter^{-1} ammonium chloride; 0.35 g liter^{-1} potassium phosphate, dibasic; 6 g liter^{-1} Tris base, with the pH adjusted to 7.5 with hydrochloric acid] [22a], 1 mM $MgSO_4$) was used to resuspend cell pellets for microscopy. Competent cells were prepared essentially as described previously by Msadek et al. (42a), replacing GE medium with MD medium (100 mM potassium phosphate [pH 7.5], 4 mM trisodium citrate, 2% [wt/vol] glucose, 11 mg liter^{-1} ferric ammonium citrate, 0.25% [wt/vol] potassium aspartate, 3 mM magnesium sulfate, 50 μg ml^{-1} L-tryptophan, and 50 μg ml^{-1} L-phenylalanine). Antibiotic resistance markers were selected by using the following drug concentrations: 5 μg ml^{-1} chloramphenicol for *cat*, 0.5 μg ml^{-1} erythromycin and 12.5 μg ml^{-1} lincomycin for *erm*, and 100 μg ml^{-1} spectinomycin for *spc*.

The strains used in this study are listed in Table 1, and plasmids and oligonucleotides used in this study are listed in Tables S1 and S2 in the supplemental material. Strains are derivatives of strain JH642 and have the common genotype *trpC2 pheA1* (6). The following alleles were described previously: Δ*sda* and *amyE*::(P_{spoIIE}-*lacZ cat*) (9) and *flgM*Δ80 (39). See the supplemental material for details on the construction of strains and plasmids.

Sporulation and β-galactosidase assays. Sporulation frequencies were determined as the ratio of heat-resistant (80°C for 20 min) CFU to total CFU. Assays were done 20 to 24 h after the end of exponential growth. β-Galactosidase-specific activity {[ΔA_{420} per min per ml of culture per optical density at 600 nm (OD_{600})] × 1,000} was determined as described previously (38). Sample absorbances at 420 nm were measured after pelleting of cell debris.

Immunoblot analysis. Two-milliliter samples were taken at the indicated times and pipetted into tubes on ice containing 200 μl of 2% (wt/vol) sodium azide–50 mM EDTA (pH 8.0) prior to pelleting of the cells by centrifugation. Cells were washed with phosphate-buffered saline (pH 7.5), resuspended in 50 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.5 mg ml^{-1} lysozyme, 100 U ml^{-1} DNase I, 20 μg ml^{-1} RNase A, 0.5% [vol/vol] protease inhibitor cocktail [P-8849; Sigma]), and incubated for 5 min at 37°C. Cell debris was pelleted by centrifugation at 20,000 × *g* for 10 min, and the protein concentrations of the supernatants were determined by a Bradford assay (Bio-Rad) without transferring the supernatants into fresh tubes. Laemmli SDS loading buffer was then added to a final concentration of 1×, and the cell pellets were resuspended and mixed with the supernatants by vortexing. In this way, the samples consisted of total cellular protein, with the amount of each sample loaded per lane based on the soluble protein concentration. Samples were separated on 13.8% SDS Tris-Tricine polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were probed with polyclonal rabbit antibodies raised against Sda (1:20,000 dilution) or σ^A (1:25,000 dilution; kindly provided by Masaya Fujita and Richard Losick), followed by incubation with a 1:10,000 dilution of goat anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad). Detection was performed by chemiluminescence using the ECL system (Amersham) and exposure to X-ray film (Kodak BioMax Light film). Images were scanned from film by using a flatbed scanner (Epson) and Adobe Photoshop, and the scanned immunoblots were quantified by using ImageQuant software (Amersham).

Microscopy. Samples were taken for fluorescence microscopy (0.5 to 1.0 ml) at the times indicated in the figures. Cells were pelleted by centrifugation for 1 min at 5,000 × *g* and resuspended in a small amount (~100 μl) of T-base medium. Cell membranes were visualized by using the fluorescent dye FM4-64 (final concentration, 5 μg ml^{-1}; Invitrogen), and DNA was visualized by using DAPI (4′,6-diamidino-2-phenylindole) (1 μg ml^{-1}). Samples were immobilized on 1% agarose pads and visualized by using a Zeiss AxioImager M1 microscope fitted with an Orca-ER charge-coupled-device (CCD) camera (Hamamatsu). The following Zeiss filter sets were used: 43 (FM4-64), 44 (green fluorescent protein [GFP]), and 49 (DAPI). Images were collected and processed by using OpenLAB 5 (Improvision).

Microarray data accession number. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (15a) and are accessible through GEO series accession number GSE22296 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22296).

TABLE 1. Strains used in this study

Strain	Genotype	Reference
JH642	*trpC2 pheA1*	6
168	*trpC2*	8
AM48	*amyE*::(P_{yneA}-*lacZ cat*) *trpC2 pheA1*	
BB424	*amyE*::(P_{ywlC}-*lacZ cat*) *trpC2 pheA1*	
BB494	*amyE*::(P_{sda}-*lacZ cat*) *trpC2 pheA1*	
BB668	Δ*sda trpC2 pheA1*	9
BB825	*amyE*::(P_{spoIIE}-*lacZ cat*) *trpC2 pheA1*	51
BB827	Δ*sda amyE*::(P_{spoIIE}-*lacZ cat*) *trpC2 pheA1*	51
BB1185	*flgM*Δ80 *trpC2 pheA1*	39
KM81	*amyE*::(P_{spoIIE}-*gfp spc*) *trpC2 pheA1*	
KM96	Δ*sda amyE*::(P_{spoIIE}-*gfp spc*) *trpC2 pheA1*	
SH350	*thrC*::(P_{tagC}-*lacZ erm*) *trpC2 pheA1*	
SH351	Δ*sda thrC*::(P_{tagC}-*lacZ erm*) *trpC2 pheA1*	
SH430	*flgM*Δ80 *aroD*::*erm trpC2 pheA1*	
SH455	*flgM*Δ80 Δ*sda trpC2 pheA1*	
SH493	Δ*sda amyE*::(P_{yneA}-*lacZ cat*) *trpC2 pheA1*	
SH507	*amyE*::(P_{dnaA}-*lacZ cat*) *trpC2 pheA1*	
SH517	*amyE*::(P_{katA}-*gfp spc*) *trpC2 pheA1*	
SH536	*amyE*::(P_{katA}-*lacZ cat*) *trpC2 pheA1*	

RESULTS

Induction of the replication checkpoint protein Sda inhibits sporulation when cells are grown in LB medium. We were led to examine the effects of manganese limitation on the transcriptional regulation of *sda* and other DnaA-dependent genes after we observed that the transcription of an *sda-lacZ* transcriptional fusion was strongly induced when cells were plated onto LB agar plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), whereas expression was off on "rich" sporulation media such as Difco sporulation medium (DSM) (data not shown). The key difference turned out to be the presence or absence of manganese: *sda-lacZ* expression was off when cells were plated onto LB agar supplemented with 100 μM manganese chloride, the concentration present in our laboratory's inherited recipe for Difco sporulation medium (the original recipe specifies 10 μM $MnCl_2$ [52]). We conjec-

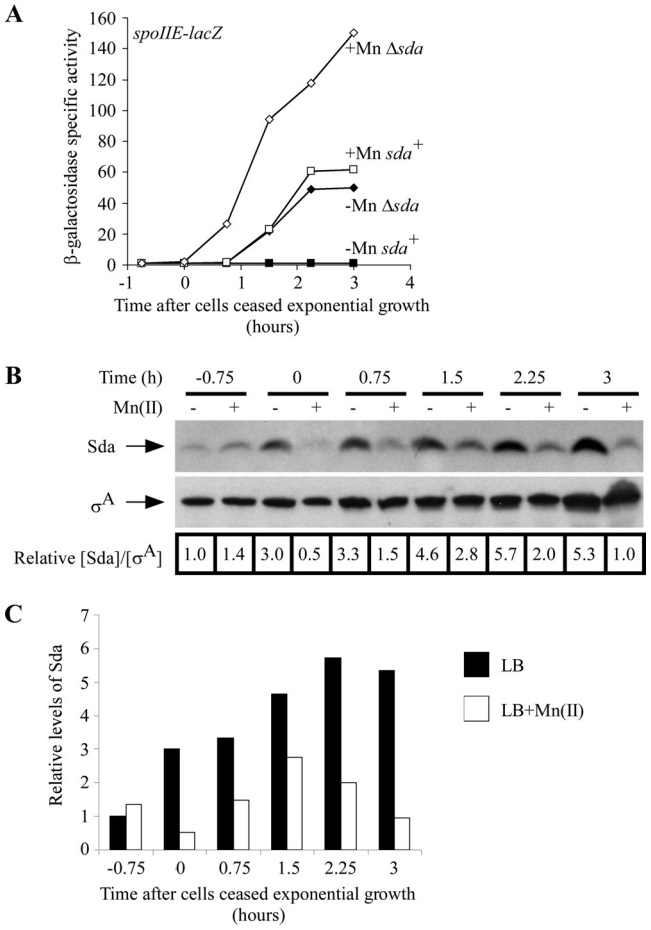

FIG. 1. Sda is induced and prevents sporulation-specific gene expression when cells are grown in LB medium unless manganese is added. (A) The *sda* replication checkpoint prevents sporulation-specific gene expression in LB medium in the absence of supplemented manganese. Cells were grown in LB medium at 37°C to an OD_{600} of 0.3 to 0.5; diluted back to an OD_{600} of 0.02 into fresh, prewarmed LB medium or LB medium supplemented with 100 μM $MnCl_2$; and then grown until 3 h after exit from exponential growth. The initiation of sporulation was monitored by using a *lacZ* transcriptional fusion to the sporulation-specific gene *spoIIE*. Sporulation frequencies were determined 20 to 24 h after cells ceased exponential growth (Table 2). Filled symbols, LB medium without Mn(II); open symbols, LB medium with Mn(II); squares, BB825 (*sda*$^+$ *amyE*::P_{spoIIE}-*lacZ*); diamonds, BB827 (Δ*sda amyE*::P_{spoIIE}-*lacZ*). (B) Levels of Sda increase as cells are grown into stationary phase in LB medium without additional manganese. Samples were taken at the indicated times from the cultures grown as described above (A), and Sda levels were determined by quantitative immunoblotting as described in Materials and Methods. Membranes were stripped and reprobed to determine σ^A levels as an internal control for sample preparation and loading. The numbers under each lane indicate the Sda/σ^A ratio normalized to the ratio of the LB [without Mn(II)] mid-log-phase sample. Five micrograms of total protein was loaded onto each lane, run on a 13.8% SDS Tris-Tricine polyacrylamide gel, transferred onto PVDF membranes, and then probed to determine protein levels. (C) Graph of the data in B.

tured that the induction of *sda* might account in part for the long-standing observation that *Bacillus subtilis* and other *Bacillus* spp. sporulate poorly on peptone- or tryptone-containing rich medium unless the medium is supplemented with manganese (10; for a review, see references 31 and 48).

To test this hypothesis, we grew *sda*$^+$ and Δ*sda* strains in liquid LB medium with or without 100 μM $MnCl_2$, monitoring sporulation-specific gene expression using a *lacZ* transcriptional fusion to the promoter for a gene that is induced at high levels of phosphorylated Spo0A (Spo0A~P), *spoIIE* (18, 46), and monitoring Sda expression levels by quantitative immunoblotting (Fig. 1; data from a representative experiment are shown). We found that the expression of *spoIIE-lacZ* was induced in *sda*$^+$ cells shortly after cells ceased exponential growth in LB medium with 100 μM $MnCl_2$ but that expression was inhibited when cells were grown in LB medium lacking manganese (Fig. 1A). In contrast, *spoIIE-lacZ* expression was induced in Δ*sda* cells in the presence or absence of manganese, suggesting that Sda contributes to the inhibition of sporulation-specific gene expression in LB medium lacking manganese. Concentrations of manganese as low as 1 μM were sufficient to restore *spoIIE-lacZ* induction in *sda*$^+$ cells entering stationary phase (see Fig. S1 in the supplemental material). The relative abundance of Sda increased approximately 2- to 5-fold in LB medium compared to that in LB medium plus 100 μM $MnCl_2$ as cells ceased exponential growth (Fig. 1B and C). Sda levels transiently increased to a similar extent following UV irradiation, delaying the initiation of sporulation (51).

We incubated the cultures for an additional 20 to 24 h following the cessation of exponential growth and determined the fraction of cells in each culture that had formed heat-resistant spores (Table 2). The sporulation frequency of the *sda*$^+$ strain was ~25-fold lower than that of the Δ*sda* strain when cells were grown in LB medium lacking manganese, whereas both strains sporulated at high frequencies in LB medium with Mn(II) (Table 2). Thus, the induction of Sda inhibits sporulation-specific gene expression and spore development when cells are grown in LB lacking added manganese. We note that the sporulation frequency of the Δ*sda* strain was ~400-fold higher when grown in LB medium with Mn(II) than when grown in LB medium alone and, similarly, that *spoIIE-lacZ* expression was nearly 2-fold higher when the Δ*sda* strain was grown in LB medium plus Mn(II) than when grown in LB medium alone (Fig. 1A). Based on these data, it appears that manganese limitation inhibits sporulation through both *sda*-dependent and *sda*-independent mechanisms.

Since *spoIIE-lacZ* expression in the Δ*sda* strain was reduced only 2-fold when cells were grown in LB medium versus LB medium plus Mn(II), whereas the sporulation frequency was reduced ~400-fold, we used fluorescence microscopy to determine the fraction of cells in each culture that initiated sporulation-specific gene expression and

TABLE 2. Sda reduces the efficiency of sporulation when cells are grown in LB medium lacking manganese[a]

Relevant genotype	Strain	Mn(II) concn (μM)	Total CFU ml^{-1}	No. of spores ml^{-1}	Sporulation frequency[b]
Wild type	BB825	0	1.2×10^9	5.9×10^4	4.9×10^{-5}
Δ*sda*	BB827	0	6.5×10^8	8.4×10^5	1.3×10^{-3}
Wild type	BB825	100	9.7×10^8	5.3×10^8	0.55
Δ*sda*	BB827	100	8.5×10^8	4.6×10^8	0.54

[a] Data are from the same experiment shown in Fig. 1. Similar sporulation data were obtained in 10 additional independent experiments (data not shown).
[b] The sporulation frequency is the ratio of spores ml^{-1} to total CFU ml^{-1}.

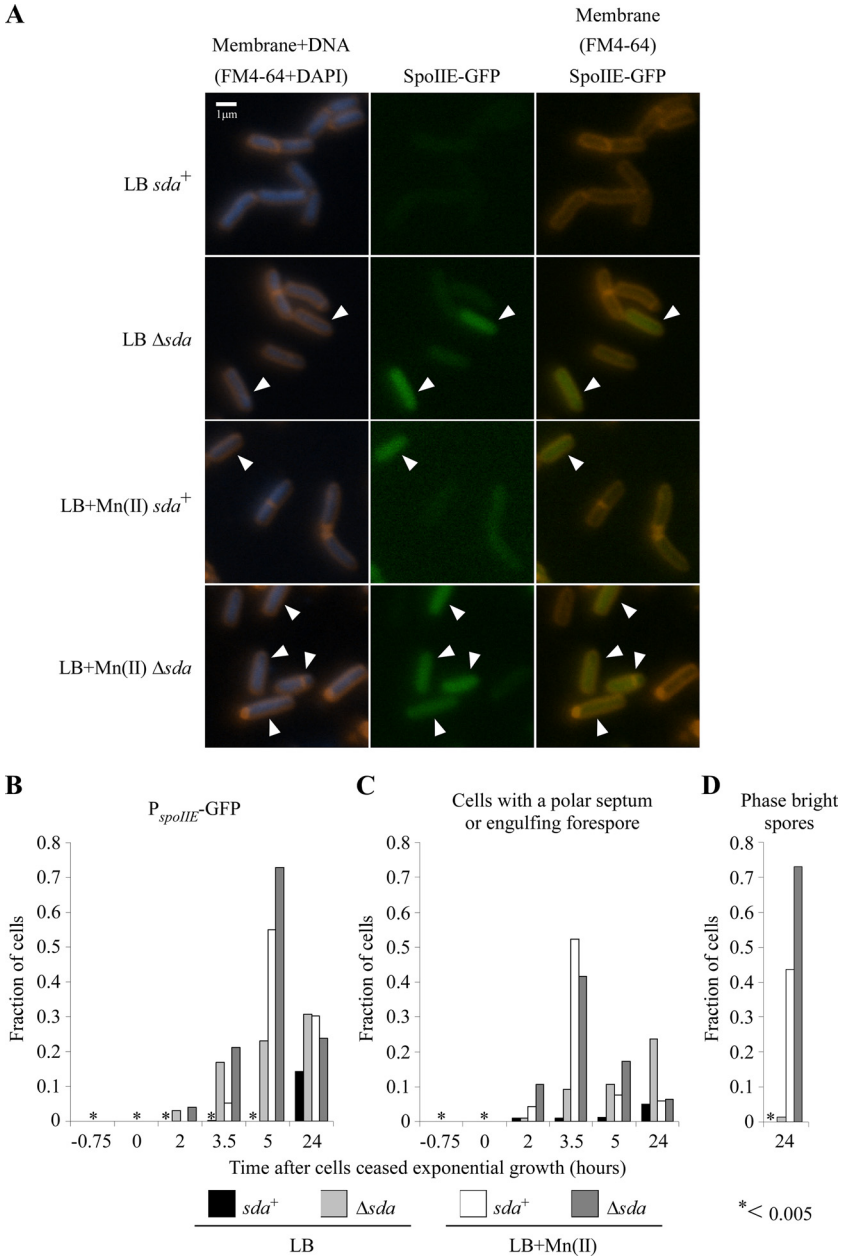

FIG. 2. Sda prevents sporulation-specific gene expression in LB medium lacking supplemental manganese. (A) Polar division and sporulation-specific gene expression were assessed by fluorescence microscopy. Cells were grown at 37°C in LB medium alone or LB medium supplemented with 100 μM $MnCl_2$ until 24 h after inoculation. The initiation of sporulation was monitored by using a GFP transcriptional fusion to the sporulation-specific gene *spoIIE*. At the following time points, samples were collected and examined under a microscope: 45 min prior to the onset of stationary phase, at the onset of stationary phase, 120 min after the onset of stationary phase, 210 min after the onset of stationary phase, 300 min after the onset of stationary phase, and 24 h after sample inoculation. Cells were stained with the fluorescent dyes FM4-64 and DAPI to stain membranes and DNA, respectively. Representative images from the time point at 210 min after the onset of stationary phase are shown, with those cells expressing *spoIIE* indicated by arrowheads. Strains used were KM81 (*sda*$^+$ *amyE*::P_{spoIIE}-*gfp*) and KM96 (Δ*sda amyE*::P_{spoIIE}-*gfp*). (B to D) Quantitation of microscopy time course. Cells were grown as described above (A), samples were taken at the indicated times, and microscopy images were scored for *spoIIE-gfp* expression, polar septa, and phase-bright spores. More than 200 cells were scored for each sample at each time point. At least two independent experiments were performed; the results from one representative experiment are shown. An asterisk indicates that the fraction of cells is less than 0.005. (B) Quantitation of cells expressing *spoIIE-gfp*. (C) Quantitation of cells that have polar septa. (D) Quantitation of phase-bright spores produced.

successfully proceeded through the first major morphological step of spore development, division at the cell pole. We monitored sporulation-specific gene expression using a *spoIIE-gfp* transcriptional fusion and polar septation using the fluorescent dye FM4-64, which stains the cytoplasmic membrane and the polar division septum prior to the completion of spore engulfment (47) (Fig. 2A to C). After incubating the cultures overnight, we determined the fraction of phase-bright spores that had formed (Fig. 2D).

Five hours after the onset of stationary phase, *spoIIE-gfp* expression was induced in approximately 60 to 80% of the population when *sda*$^+$ and Δ*sda* strains were grown in LB medium with Mn(II) (Fig. 2A and B; arrowheads indicate cells expressing GFP from the *spoIIE* promoter). When strains were grown in LB medium alone, the Δ*sda* strain showed increasing *spoIIE-gfp* expression over time, with approximately 25% of the population expressing GFP 5 h after the onset of stationary phase. In contrast, little or no GFP expression was observed in the *sda*$^+$ strain at the 5-h time point. Thus, the fraction of cells that induced *spoIIE-gfp* expression directly paralleled the levels of *spoIIE-lacZ* expression shown in Fig. 1. A similar pattern was observed when we scored the fraction of cells with polar septa or engulfing forespores (Fig. 2C) except that the frequencies of polar septation peaked at 3.5 h rather than 5 h. The subsequent decrease by 5 h reflects the completion of spore engulfment, when FM4-64 no longer stains the forespore membrane. The frequencies of polar septation were somewhat lower for the Δ*sda* strain in both LB medium and LB medium with Mn(II) than for the fraction of cells expressing GFP at 5 h. By 24 h, 40 to 70% of the *sda*$^+$ and Δ*sda* cells grown in LB medium plus Mn(II) had formed phase-bright spores, whereas when cells were grown in LB medium lacking manganese, fewer than 2% of the Δ*sda* cells formed phase-bright spores, and no phase-bright spores were observed in the *sda*$^+$ culture (Fig. 2D). These data parallel the sporulation frequencies shown in Table 2. Thus, Δ*sda* cells grown in LB medium proceed through the early stages of sporulation at high frequencies, similar to strains grown in LB medium plus Mn(II), but a large fraction of cells subsequently arrested development prior to the formation of phase-bright or heat-resistant spores. Likewise, a small fraction of *sda*$^+$ cells expressed *spoIIE-gfp* and had divided at one cell pole by the 24-h time point (Fig. 2B and C), but the level of production of phase-bright spores was too low for detection (Fig. 2D). These data support the conclusion that manganese is required both for the efficient initiation of sporulation, to prevent induction of Sda, and for efficient progression through one or more stages of development following polar septation.

Previously, Freese and coworkers established that the activity of the glycolytic enzyme phosphoglycerate mutase was strictly dependent on Mn(II) and that cells of *B. subtilis* were unable to sporulate in LB medium with glucose due to the accumulation of the enzyme's substrate during glycolysis, 3-phosphoglycerate (3-phosphyglyceric acid [3-PGA]) (45, 53). Supplementation of the medium with manganese prevents the accumulation of 3-PGA and restores efficient sporulation (45, 53). Supplementation of the medium with L-malate also prevents 3-PGA accumulation and restores efficient sporulation by providing a route for metabolizing 3-PGA via the tricarboxylic acid (TCA) cycle independently of phosphoglycerate mutase (53). To see if a similar mechanism of sporulation inhibition and 3-PGA accumulation due to insufficient manganese was involved in the block to sporulation in LB medium, we looked at *spoIIE-lacZ* expression in cells grown in LB medium that had been supplemented with L-malate and compared that pattern of expression to those of cells grown in LB medium and LB medium with additional manganese. We found no difference in the patterns of sporulation-specific gene expression or the production of heat-resistant spores with the addition of L-malate (data not shown, and see Fig. S2 in the supplemental material), indicating that this mechanism is not likely to be involved in the block to sporulation that we observed with LB medium without additional manganese.

Changes in DnaA-dependent gene expression suggest that the levels of active DnaA are higher when cells are grown in LB medium lacking supplemental manganese. A prolonged expression of Sda is induced by conditions that perturb replication initiation or elongation or that induce DNA damage (4, 7, 9, 20, 30, 43, 51, 54). Thus, the data presented above suggest that cells grown in LB medium without supplemental manganese are experiencing some kind of replication stress.

Several conditions that block replication elongation activate *sda* transcription by activating the DnaA-dependent pathway and by inducing the SOS response. However, *sda* is also induced by a variety of conditions that activate DnaA alone without inducing the SOS response (9, 20, 30, 43, 54). We sought to determine if one or both of these pathways were activated when cells were grown in LB medium lacking manganese.

We used *lacZ* transcriptional fusions to the promoters of two SOS-inducible genes, *tagC* (*dinC*) and *yneA*, to determine if DNA damage could be responsible for the activation of *sda* expression (12, 34). Neither reporter was detectably induced on LB medium or LB medium plus Mn(II) indicator plates in either the *sda*$^+$ or Δ*sda* strain background (strains AM48 [*sda*$^+$ *amyE*::P_{yneA}-*lacZ*], SH493 [Δ*sda amyE*::P_{yneA}-*lacZ*], SH350 [*sda*$^+$ *thrC*::P_{tagC}-*lacZ*], and SH351 [Δ*sda thrC*::P_{tagC}-*lacZ*] [data not shown]). *yneA* is a particularly sensitive marker, since it is induced at concentrations of DNA-damaging agents that do not induce *tagC* (4; A. Mo and W. F. Burkholder, unpublished data). Therefore, since no expression was seen from either reporter, we concluded that DNA damage resulting in the activation of the SOS response was not a major factor in the activation of *sda* expression in LB medium.

In order to determine if *sda* transcription was activated via the DnaA-dependent pathway, we used *lacZ* transcriptional fusions to monitor the expression of two genes that are repressed when active DnaA accumulates, *dnaA* itself and *ywlC* (7, 20, 30, 44). The expression of each reporter gene was monitored over time as samples were grown in LB medium and LB medium with additional manganese until 3 h after the cessation of exponential growth (Fig. 3A). Looking at levels of active DnaA during exponential growth using the *dnaA* reporter, we found that the reporter gene expression level was higher in LB medium with manganese added than in LB medium until approximately 2 h after the onset of stationary phase, when the levels were indistinguishable. This pattern was consistent with higher levels of active DnaA being present in LB medium without additional manganese than in LB medium with additional manganese. The *ywlC* reporter was used to examine the levels of active DnaA during stationary phase, and again, expression was seen only starting 2 h after cells had ceased exponential growth when manganese was added to the medium, indicating that levels of active DnaA were higher in LB medium without manganese than in LB medium with manganese.

To rule out the possibility that the differences in reporter gene expression were due to long-term effects of growing cells in the presence or absence of manganese, we measured the

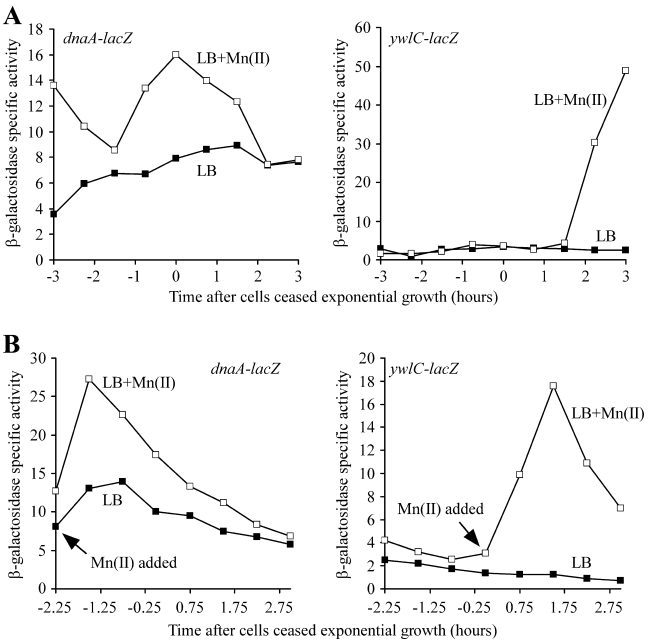

FIG. 3. The expression patterns of DnaA-dependent reporter genes suggests that the levels of active DnaA are elevated in LB medium lacking supplemental manganese. (A) *dnaA*-dependent reporter genes show higher levels of expression when grown in LB medium with supplemented manganese than when grown in LB medium without additional manganese. Cells were grown in LB medium to an OD_{600} of 0.3 to 0.5, diluted back into fresh LB medium alone or LB medium supplemented with 100 μM $MnCl_2$, and then grown at 37°C until 3 h after exit from exponential growth. To monitor levels of active DnaA during exponential growth, a *lacZ* transcriptional fusion to *dnaA* was used. Levels of active DnaA during stationary phase were monitored by using a *lacZ* transcriptional fusion to *ywlC* (see the text for details). Three independent experiments were performed; the results from one representative experiment are shown. Strains used were SH507 (*sda*$^+$ *amyE*::P_{dnaA}-*lacZ*) and BB424 (*sda*$^+$ *amyE*::P_{ywlC}-*lacZ*). (B) *dnaA*-dependent reporters show higher expression levels after manganese is added to cultures growing in LB medium than in control cultures of LB medium without additional manganese. Cells were grown in LB medium at 37°C. At the indicated times, 100 μM $MnCl_2$ was added to half of each culture, while the other half received no supplementation. The same reporters as those detailed above (A) were monitored for expression until 3 h after cells had ceased exponential growth. Three independent experiments were performed; the results from one representative experiment are shown. Strains used were SH507 (*sda*$^+$ *amyE*::P_{dnaA}-*lacZ*) and BB424 (*sda*$^+$ *amyE*::P_{ywlC}-*lacZ*).

acute effect of adding manganese to cultures grown initially in LB medium alone on gene expression (Fig. 3B). Strains were grown in LB medium to mid-exponential phase (for the *dnaA* reporter strain) and the onset of stationary phase (for the *ywlC* reporter strain). The cultures were then split, adding 100 μM manganese to one of the two flasks for each strain. Consistent with our previous results, the addition of manganese induced the expression of both the *dnaA-lacZ* and the *ywlC-lacZ* transcriptional fusions. These data indicate that levels of active DnaA are higher in LB medium lacking supplemental manganese.

Since the SOS response to DNA damage is not induced, activated DnaA might accumulate due to the misregulation of replication initiation or to changes in replication fork processivity that are independent of significant DNA damage, helicase uncoupling, or replication fork collapse. Conditions that perturb replication initiation and induce *sda* expression affect the chromosome copy number by causing asynchronous initiation (so that cells have an odd number of origins of replication rather than 1, 2, 4, and 8, etc.), by increasing the number of origins per cell (overinitiation), or by inhibiting replication initiation (9, 40, 43). We asked if the presence or absence of manganese affected replication synchrony or chromosome copy numbers by growing *sda*$^+$ and Δ*sda* strains in LB medium and LB medium plus 100 μM Mn(II) at 26°C and taking samples during exponential growth and 3 h after cells had ceased exponential growth (T_3). Cells were then incubated in the presence of chloramphenicol to block replication initiation and cell division for several hours to allow ongoing rounds of DNA replication to be completed, and the relative DNA content per cell was determined by flow cytometry (see the supplemental material). Since cells of *B. subtilis* normally grow in chains during exponential growth, we used strains that lack the *flgM* gene (BB1185 [*sda*$^+$ *flgM*Δ80] and SH455 [Δ*sda* *flgM*Δ80]), which rapidly separate into single cells during exponential growth due to the overexpression of the autolysins that promote cell separation (39). As expected for cells entering the stationary phase, the chromosome copy number in both strains decreased from predominantly 2 to 4 chromosome equivalents to 1 to 2 chromosome equivalents between the mid-exponential time point and T_3 (Fig. 4). There were slight differences in the chromosome copy number profiles between the *sda*$^+$ and Δ*sda* strains grown in the presence of manganese at T_3, but none of the profiles suggested that there were significant differences in replication synchrony and chromosome copy number between strains grown in the presence or absence of manganese (Fig. 4). Although we cannot rule out the possibility that *sda* expression is induced due to a subtle perturbation in replication initiation, it seems most likely that the level of DnaA activity is higher in LB medium lacking manganese due to effects on replication fork progression that do not significantly activate the SOS response.

Microarray analysis. To better understand how manganese limitation might increase the levels of active DnaA, we performed whole-genome expression profiling using 65-mer oligonucleotide microarrays (Genosys). *sda*$^+$ and Δ*sda* strains were grown in LB medium and LB medium supplemented with 100 μM manganese, and RNA was purified from samples collected at three time points: mid-exponential phase (OD_{600} of 0.3 to 0.5; "T_m"), when cells ceased exponential growth (T_0), and 3 h after cells ceased exponential growth (T_3) (GEO accession number for microarray data, GSE22296; see the supplemental material for experimental details).

The results from the microarray analysis indicated that the number of differentially expressed genes increased over time under these conditions (see Table S8 in the supplemental material for the fold differences in relative gene expression organized by regulon and Table S9 for complete microarray data and statistics). At the first two time points (T_m and T_0), the differences in gene expression were due mainly to the presence or absence of manganese. At T_3, when cells initiated spore development in LB medium supplemented with manganese, a large number of genes were differentially expressed, depending on the presence or absence of manganese, the presence or absence of *sda*, or both.

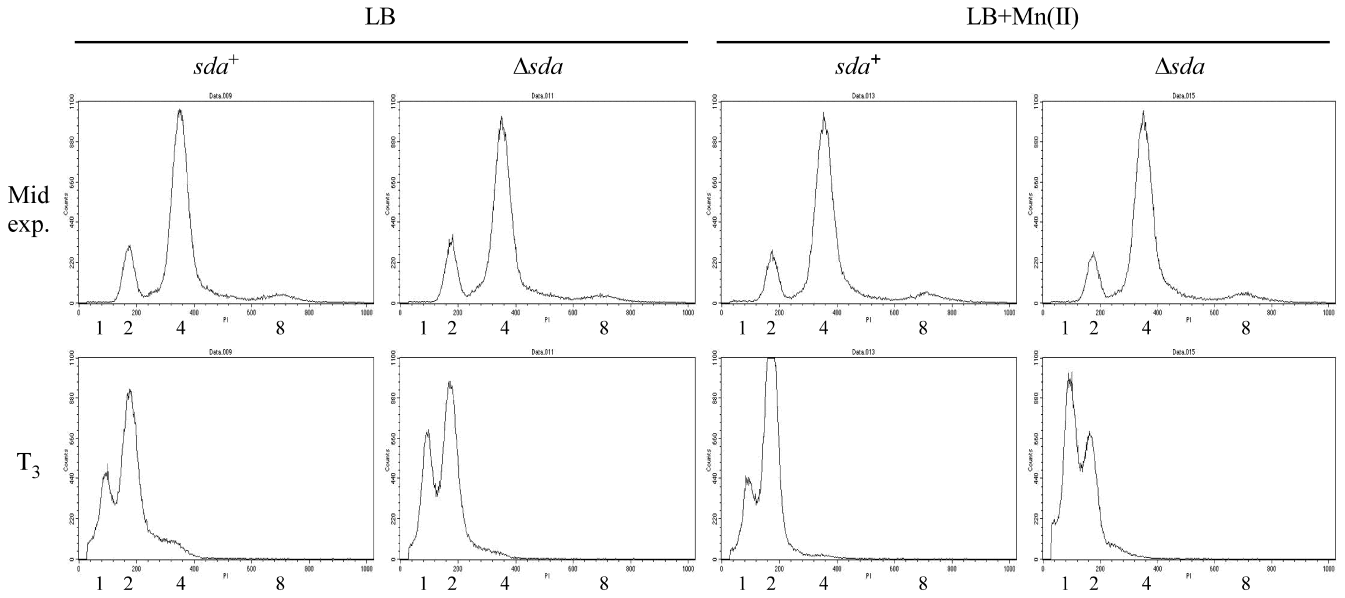

FIG. 4. The presence or absence of manganese in LB medium has little or no effect on chromosome copy number or the synchrony of replication initiation. Cells were grown in LB medium or LB medium plus 100 μM $MnCl_2$ at 26°C to an OD_{600} of 0.3 to 0.5 for mid-exponential-phase samples or 37°C until 3 h after the onset of stationary phase for T_3 samples. At the indicated times, a 5-ml aliquot was removed and incubated with 200 μg/ml of chloramphenicol for 6 h to inhibit further rounds of replication from initiating but to allow current rounds to finish. Cells were then fixed in ethanol, washed twice with 200 mM Tris-HCl at pH 7.5, and subsequently incubated with 100 μg/ml RNase A for 1 h at 60°C. Cells were then stained with propidium iodide to a final concentration of 5 μg/ml and diluted (if necessary), and 10,000 cells were analyzed for DNA content with a flow cytometer. The numbers under the flow cytometry profiles correspond to chromosomal equivalents. Strains used were BB1185 (*sda*$^+$ *flgM*Δ80) and SH455 (Δ*sda flgM*Δ80).

The microarray data supported our conclusions that the induction of *sda* was due to the activation of the DnaA-dependent signaling pathway but not to the SOS response to DNA damage. Little or no induction of SOS-inducible genes (LexA-repressed genes and prophage genes) was observed at any time point when cells were grown in the absence of manganese (see Tables S8 and S9 in the supplemental material). *dnaA* expression was significantly reduced at T_0 in both strains when cells were grown in the absence of manganese (or, as shown in Table S3, expressed at significantly higher levels in the presence of manganese), paralleling our data obtained using the *dnaA-lacZ* transcriptional fusion (Fig. 3). Similarly, one of the operons positively regulated by DnaA, *yurY-yurX-csd-nifU-yurU*, encoding orthologs of the *E. coli* Fe-S assembly complex subunits SufB, SufC, and SufD, was expressed at significantly higher levels in the absence of manganese at T_0 (Table S3). No significant differences in expression were observed for *ywlC* at T_3, which probably reflects the lower sensitivity of the array data under these growth conditions.

Several DnaA-regulated operons were differentially expressed at T_0 in the direction opposite of that expected if changes in expression were due solely to activated DnaA, including *ykuNOP*, encoding two flavodoxins, and *dhbACEBF*, encoding enzymes required for the biosynthesis of the iron siderophore bacillibactin (see Table S3 in the supplemental material). The *ykuNOP* and *dhbACEBF* operons are repressed by Fur and were expressed at significantly higher levels in both strains in the absence of manganese. Several other Fur-repressed operons were also expressed at significantly higher levels in the absence of manganese (Table S4). Many, like *dhbACEBF*, are involved in siderophore production and iron-siderophore uptake. These data are consistent with previous observations that high concentrations of Mn(II) repress the Fur regulon via PerR (17, 22).

The expressions of many sporulation-specific genes were inhibited at T_3 when *sda*$^+$ cells were grown in the absence of manganese (see Table S5 in the supplemental material). These data parallel the results described above, obtained using the *spoIIE-lacZ* and *spoIIE-gfp* transcriptional fusions. In addition to *spoIIE*, several other genes are induced by high levels of Spo0A~P during the initiation of sporulation (18): *spoIIA* (*spoIIAA*, *spoIIAB*, or *sigF*), *spoIIG* (*spoIIGA*, *sigE*, or *sigG*), *racA*, *yttP*, *sinI*, and *sirA* (*yneE*), which encodes a protein that inhibits further rounds of replication in sporulating cells by inhibiting DnaA (49, 55). The levels of expression of all of these genes were significantly lower ($q \leq 0.005$) at T_3 in *sda*$^+$ strains grown in the absence of manganese based on the following ratios of relative gene expression from the four growth conditions: [*sda*$^+$ strain in LB medium plus Mn(II)]/(*sda*$^+$ strain in LB medium) ratio of ≥1.5-fold ($\log_{10}$) and (*sda*$^+$ strain in LB medium)/(Δ*sda* strain in LB medium) ratio of ≤1.5-fold (Table S5). As observed with the *spoIIE* transcriptional fusions, the levels of expression of many sporulation genes were also significantly higher in the Δ*sda* strain grown in LB medium plus Mn(II) than in LB medium.

Only 13 genes were found to have significant changes in gene expression at the first time point (T_m) (see Table S6 in the supplemental material). Of these genes, 11 were significantly downregulated in both strains at all three time points when cells were grown in LB medium plus Mn(II) (Table S6). Con-

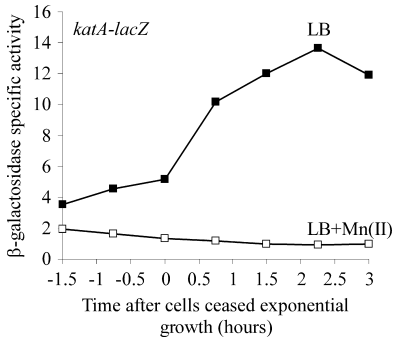

FIG. 5. *katA* expression is induced when cells are grown in LB medium lacking supplemental manganese. Cells were grown in LB medium at 37°C to an OD_{600} of 0.3 to 0.5; diluted back to an OD_{600} of 0.02 into fresh, prewarmed LB medium or LB medium supplemented with 100 μM $MnCl_2$; and then grown until 3 h after exit from exponential growth. Catalase levels were monitored by using a *lacZ* transcriptional fusion to *katA*. Filled symbols, LB medium without Mn(II); open symbols, LB medium with Mn(II). Strain SH536 (*sda*$^+$ *amyE*::P_{katA}-*lacZ*) was used.

sistent with the growth conditions, these included two operons encoding manganese transport systems (*mntABCD* and *mntH*), regulated by MntR. The gene encoding vegetative catalase (*katA*), a member of the PerR regulon, was also expressed at significantly lower levels in LB medium with Mn(II) at all three time points. The PerR regulon is most closely associated with the response to inorganic peroxides and is induced during exposure to peroxide and superoxide stress. Several other PerR-regulated operons were expressed at significantly lower levels in LB medium plus Mn(II) at T_0 and T_3: *ahpCF*, encoding alkyl hydroperoxide reductase; *hemAXCDBL*, required for heme biosynthesis; and *perR* itself (Table S7). The manganese-dependent regulation of MntR- and PerR-dependent gene expression has been well characterized (11, 17, 22, 24). Interestingly, but for reasons that are not clear, the PerR-regulated gene encoding the iron-sequestering protein MrgA was expressed at lower levels at T_3 but only for the Δ*sda* strain (Table S7). These data suggest that cells may be undergoing oxidative stress when grown in the absence of manganese.

Oxidative stress response genes are induced in cells grown to stationary phase in LB medium lacking supplemental manganese. To corroborate the finding that *katA* was induced when cells were grown in LB medium lacking manganese, we monitored *katA* expression using a *lacZ* transcriptional fusion and grew cells in LB medium in the presence or absence of 100 μM manganese (Fig. 5). Consistent with the microarray data, the level of expression of *katA* increased over time, particularly after the onset of stationary phase, when cells were grown in LB medium. Little or no expression of *katA* was observed when cells were grown in LB medium with Mn(II). The stationary-phase induction of *katA* and the strong repression of *katA* transcription by manganese have been extensively characterized (11, 17, 22, 24). Microscopy experiments using a *katA-gfp* transcriptional fusion revealed that *katA-gfp* expression was induced in the entire population when cells were grown in LB medium, whereas little or no GFP fluorescence was observed when cells were grown in LB medium with Mn(II) (strain SH517 [*amyE*::P_{katA}-*gfp*] [data not shown]). Taken together with the microarray results, the expression pattern for *katA* indicates that cells may be experiencing oxidative stress when grown in LB medium lacking supplemented manganese, which in turn might be responsible for perturbing DNA replication.

DISCUSSION

Based on our results, we conclude that changes in DnaA-dependent gene expression contribute to the transcriptional and developmental responses of *B. subtilis* to manganese-limited growth in LB medium. Cells of *B. subtilis* sporulate poorly in LB medium in part because of the activation of the Sda checkpoint, which delays or inhibits sporulation until DNA replication has been completed. Sda expression is induced and prevents sporulation-specific gene expression in LB medium unless the medium is supplemented with additional manganese (Fig. 1B and C). Cells lacking *sda* initiate spore development at a high frequency, inducing sporulation-specific gene expression and dividing at the cell pole (Fig. 1A and 2, and see Table S4 in the supplemental material). However, following polar septation, Δ*sda* cells frequently fail to form stress-resistant spores due to a requirement for manganese at some later stage of development (Table 2 and Fig. 2D). The transcription of *sda* appears to be activated solely via the DnaA-dependent pathway, which responds to perturbations of replication initiation and replication elongation, based on the following results: (i) the levels of expression of several other DnaA-dependent genes differ significantly in LB medium compared to that in LB medium plus Mn(II), consistent with the model that levels of active DnaA are higher in LB medium lacking manganese (Fig. 3 and Table S3), and (ii) the SOS response to DNA damage is not induced (based on data from *lacZ* transcriptional fusions and microarray data discussed in the text) (Tables S8 and S9). Based on flow cytometry data, it seems that replication initiation is not significantly perturbed (Fig. 4), suggesting that DnaA may activate *sda* transcription due to a perturbation of replication elongation that does not induce the SOS response. DNA replication might be perturbed due to secondary effects of reactive oxygen species, since the gene encoding vegetative catalase, *katA*, and other PerR-regulated genes involved in the oxidative stress response are expressed at significantly higher levels when cells are grown in LB medium lacking manganese (Fig. 5 and Tables S6 and S7). DNA replication might also be perturbed due to the effects of manganese limitation on one or more manganese-dependent enzymes. Regardless of the source, these data demonstrate that small perturbations in DNA replication can have large effects on development, effectively blocking endospore formation in *B. subtilis*.

The data presented here provide new insight into the long-standing observation that *Bacillus subtilis* sporulates poorly in peptone- or tryptone-containing rich medium unless the medium is supplemented with manganese (10; for a review, see references 31 and 48). However, we have only pushed the questions back by a step: how does manganese limitation in LB medium affect DNA replication or metabolism, and how do those effects lead to the activation of DnaA and DnaA-dependent changes in gene expression?

Our data indicate that manganese limitation also inhibits sporulation by *sda*-independent mechanisms (Fig. 2 and Table 2). The induction of Sda thus prevents cells from initiating

sporulation under conditions that preclude the successful completion of spore morphogenesis, as observed previously (9, 51). Although Sda is required to fully inhibit the transcription of *spoIIE* during manganese limitation, we note that manganese limitation has a small effect on the levels of *spoIIE* expression and polar septation in cells lacking *sda*, reducing the levels of *spoIIE-lacZ* expression and the fraction of cells that express *spoIIE-gfp* or that have undergone polar septation 2- to 5-fold (Fig. 1 and 2). The largest effects of manganese limitation likely occur at later stages of spore development, since cells lacking *sda* sporulate at ~400-fold-lower frequencies in LB medium than in LB medium supplemented with manganese (Table 2). Any number of mechanisms might account for the *sda*-independent effects of manganese limitation on sporulation.

Manganese is a cofactor for enzymes involved in diverse metabolic pathways, including glycolysis, the TCA cycle, amino acid utilization, and nucleotide metabolism. Some of the manganese-dependent changes in gene expression inferred from our whole-genome expression data (see Tables S8 and S9 in the supplemental material) are likely responses to the reduced activity of particular manganese-dependent enzymes in LB medium compared to LB medium with Mn(II). This same general observation has also been made regarding the whole-genome transcriptional response of *B. subtilis* grown in a defined minimal medium in the presence or absence of manganese (22). Thus, changes in energy status, deoxyribonucleotide pools, amino acid or carbohydrate utilization, or other metabolic effects may contribute alone or in combination to impede DNA replication.

Manganese also plays a major role in protecting cells from reactive oxygen species, both as a cofactor for enzymes such as manganese-dependent superoxide dismutase (SodA in *B. subtilis*) and, perhaps in complex with cellular metabolites, as a direct scavenger of reactive oxygen species (27). For instance, the high-level resistance of organisms such as *Deinococcus radiodurans* and *Lactobacillus plantarum* to radiation or oxidative stress may result in part from their exceptionally high concentrations of intracellular manganese and the direct scavenging of reactive oxygen species by manganese in complex with small molecules (2, 3, 13, 15). In cells of *B. subtilis* lacking *sodA*, manganese still confers resistance to oxidative stress in growing and sporulating cells and stimulates a hydrogen peroxide-scavenging activity that may otherwise require one or more low-molecular-weight metabolites (29).

Three of the predominant reactive oxygen species encountered by aerobically growing cells are superoxide, hydrogen peroxide, and hydroxyl radicals generated by the reaction of hydrogen peroxide with Fe(II) in the Fenton reaction (27). Superoxide and hydrogen peroxide both induce the *perR* and *fur* regulons in *B. subtilis* as well as in *E. coli* (1, 5, 17, 23, 41, 58), so the accumulation of either regulon would be consistent with the induction of *katA* and other genes in the PerR and Fur regulons that we observed (Fig. 5, and see Table S6 and S7 in the supplemental material). Hydroxyl radicals generated by the Fenton reaction are particularly reactive and cause damage to cellular proteins, lipids, stable RNA, and DNA (28). We consider it unlikely, however, that significant oxidative damage to DNA is responsible for the induction of *sda*, since we did not observe a significant induction of either SOS response genes repressed by LexA or prophage genes. We cannot rule out the possibility, however, that low levels of oxidative damage insufficient to induce the SOS response nonetheless perturb replication fork progression sufficiently to activate DnaA-dependent gene expression. The main targets of superoxide *in vivo* are enzymes with iron-sulfur clusters (most of which are key players in central metabolism) and branched-chain, aromatic, and sulfur-containing amino acids (28). Thus, oxidative stress might affect DNA replication by perturbing metabolic pathways in much the same way that manganese limitation could impair the activities of manganese-dependent enzymes.

If superoxide contributes to the oxidative stress response, it seems surprising that expression levels of the principal superoxide dismutase, SodA, were not significantly different in the presence or absence of manganese. In contrast, expression levels of a gene encoding a putative sporulation-specific Fe(II)-dependent superoxide dismutase, *sodF*, were significantly higher in the presence of manganese in both strains at T_0 and in the Δ*sda* strain at T_3. In a study characterizing the transcriptional response of *B. subtilis* to the superoxide-generating compound paraquat, the expression of *sodA* was not significantly different in the presence or absence of paraquat (41).

The results presented here underscore the point that DNA replication can be affected by a variety of environmental conditions not obviously associated with genotoxic stress and that small perturbations in DNA replication can have large developmental consequences. Consequently, mechanisms for coordinating cell cycle progression with chromosome replication and segregation, such as the Sda checkpoint pathway, are likely to play broad roles in promoting competitive fitness in natural environments.

ACKNOWLEDGMENTS

We thank Masaya Fujita and Richard Losick for anti-σ^A antiserum; Steven Biller, Kathleen Mach, and Allison Mo in the Burkholder laboratory for plasmids and strains; Robert Britton for microarray protocols and assistance with microarray printing; and Steven Biller for assistance with microarray experiments. We thank members of the Burkholder laboratory and other generous colleagues for many helpful discussions.

S.E.H. was supported by an NIH predoctoral training grant (5T32GM007276). This work was supported by National Science Foundation award ID 0744872.

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