Growth Phase Variation in Cell and Nucleoid Morphology in a *Bacillus subtilis recA* Mutant

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The major role of RecA is thought to be in helping repair and restart stalled replication forks. During exponential growth, *Bacillus subtilis recA* cells exhibited few microscopically observable nucleoid defects. However, the efficiency of plating was about 12% of that of the parent strain. A substantial and additive defect in viability was also seen for *addB* and *recF* mutants, suggesting a role for the corresponding recombination paths during normal growth. Upon entry into stationary phase, a subpopulation (~15%) of abnormally long cells and nucleoids developed in *B. subtilis recA* mutants. In addition, *recA* mutants showed a delay in, and a diminished capacity for, effecting prespore nucleoid condensation.

The RecA protein is ubiquitous among the bacteria studied and possesses one of the most conserved primary amino acid sequences. The function of the RecA protein as a mediator of homologous recombination and as a regulator of the inducible SOS response in both *Escherichia coli* and *Bacillus subtilis* has been elucidated in detail (33, 36). The primary role of RecA in *E. coli*, and perhaps in all bacteria, appears to be its participation in the housekeeping function of repairing and restarting stalled replication forks (5).

The importance of this housekeeping function is illustrated by the fact that *E. coli* PriA, which is disposable for initiation of DNA replication at *oriC*, but essential for restarting stalled replication forks, cannot be functionally inactivated without suffering a severe penalty: in growing cultures about 90% of *priA* mutant cells are dead (16, 25). Similarly, the absence of recombinational repair in *E. coli recA* mutants results in a 50% loss of viability (4). In *recA* mutants, the primary cause of death appears to be chromosomal degradation, presumably at sites of stalled DNA replication forks. About 10% of *E. coli recA* cells are anucleate, and an additional portion show signs of chromosomal degradation (29, 38).

During our analysis of site-specific recombination and chromosome dimerization in *B. subtilis*, we noted that mutations in *recA* did not completely suppress the nucleoid segregation defects associated with the inability to resolve dimeric chromosomes in *ripX* mutants (26). However, the *recA* mutation by itself had little effect on nucleoid morphology. Subsequently, we noted that about 12% of *recA* cells were viable during exponential growth and stationary phase ("viable" in this report refers to colony-forming ability). In addition, we have observed that a *B. subtilis* strain with a *recA* mutation developed a subpopulation of cells that displayed gross division defects and a loss of nucleoid integrity during the transition from exponential growth to stationary phase. These phenotypes were not seen in *E. coli recA* mutants. To our knowledge, there have been no previous reports of aberrant nucleoids in recombination-deficient strains of *B. subtilis*.

The parent strain in this study was BR151 (*trpC2 lys-3 metB10*). The ΔSPβ phage strain used (YB886) and the ΔSPβ *addB72* strain (SL7576) were obtained from the Bacillus Genetic Stock Center and are derivatives of BR151 (37). SL7131, SL7360, and SL7370 have been described previously (26). The *B. subtilis recF* strain (SL7609) was made by transformation of BR151 with pSAS28, a pBluescript plasmid containing *recF::spc* (*spc* between the *Sty*I sites within *recF*). The ΔSPβ *addB72 recF* strain (SL7611) was made by transformation of SL7576 with pSAS28. W3110 is an *E. coli recA::*Tn9 strain (34). The *recA-lacZ* strain (SL7937) was made by transformation of BR151 with YB3001 DNA (28). *comG-lacZ* (SL7952) and *comK-lacZ* (SL7954) strains were made by transformation of BR151 with BD1960 and BD1991 DNA, respectively. Bacterial cultures were grown in Luria-Bertani (LB) medium or modified Schaeffer's sporulation medium (MSSM) (21) at 37°C with shaking at 150 rpm. Media were used fresh or were stored for short periods in the dark before use; glass vessels were used throughout. Medium volume was maintained between 6 and 9% of total flask volume. *recA*, *recF*, and *addB* mutants were grown in glass flasks wrapped in aluminum foil to reduce the effects of ambient light. Mitomycin C (MMC) was used at a concentration of 20 ng ml⁻¹. Nucleoids were stained with 4′, 6-diamidino-2-phenylindole (DAPI) and were observed and photographed as described previously (26). Cell and nucleoid measurements were performed as described earlier (26). β-Galactosidase and sporulation-frequency assays were performed essentially as described previously (19). "T_0" in these studies refers to the end of exponential growth. "$T_1, T_2, T_3,$" etc., are 1, 2, and 3 h, etc., after the end of exponential growth. Plating efficiencies were derived from dilutions of exponential- or stationary-phase cultures. Dilutions were made in Spizizen's minimal salts plus 55 mM glucose, 0.02% casein hydrolysate, 0.1% yeast extract, 20 μg of tryptophan per ml, and 50 μg (each) of methionine and lysine per ml. Plating was performed in duplicate on freshly prepared LB agar, and incubation was at 37°C

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TABLE 1. Nucleoid profiles of various *rec* strains fixed during mid-exponential growth in LB medium

Strain (relevant genotype)	% with nucleoid phenotype[a]				Viability	
	Par[b]	Anucleate	Cell wall defects[c]	Total defects	CFU mg^{-1d}	Relative plating efficiency[e]
BR151 (parent)	1.2	0	0	1.2	7.46×10^8	1.0
SL7609 (*recF*::*spc*)	8.0	0.8	0.2	9.0	5.07×10^8	0.61
SL7360 (*recA*::*neo*)	4.2	0	1.8	6.0	9.27×10^7	0.12[f]
SL7370 (*recA*::*neo ripX*::*spc*)	16.0	2.4	6.0	24.4	4.15×10^7	0.056
SL7131 (*ripX*::*spc*)	26.2	1.0	0	27.2	4.28×10^8	0.57
SL7513 (ΔSPβ parent)	0.66	0	0	0.66	2.55×10^9	1.0
SL7576 (ΔSPβ *addB72*)	2.6	0.4	0	3.0	1.34×10^9	0.52
SL7611 (ΔSPβ *recF*::*spc addB72*)	5.3	0.83	0.17	6.3	8.03×10^8	0.31

[a] Minimum of 500 cells scored per strain.

[b] Par nucleoids were of two classes: those that had elongated to > 1/3 of the cell length and those in which partitioning appeared stalled. Typically the stalled nucleoids had an hourglass or dumbbell shape, but had no immediate nucleoid neighbors.

[c] Cell wall defects typically appeared as bulges in the lateral cell wall and normally contained complex nucleoid structures that may be described as "disintegrating." Examples are provided in Fig. 1E and F.

[d] The numbers of CFU were normalized by bacterial dry weight (in milligrams) with a correction table for spectrophotometric values at an optical density at 600 nm. The average of at least two experiments is shown (see text for details).

[e] Plating efficiencies are relative to the appropriate parent strain: BR151 or SL7513.

[f] The relative plating efficiency value during stationary phase for this strain was 0.16.

in the dark. Only plates with between 30 and 400 colonies were used to determine viable counts. In general, Difco components were used in the media. Preliminary experiments indicated that growth rate, cell and nucleoid morphologies, and plating efficiencies of *recA* mutants were very similar when Oxoid components were substituted for Difco components.

Nucleoid and cell morphology phenotypes of *B. subtilis rec* mutants during exponential-phase growth. In Table 1, we present an assessment of nucleoid and cell morphologies for various *rec* mutant strains and their derivatives during exponential growth. A small proportion of the *recA* mutant (SL7360) cells showed evidence of partitioning failures and cell wall irregularities. The cell wall defects observed were typically bulges in the lateral wall (Fig. 1E and F). *recA* cells with cell wall defects almost always contained complex nucleoid structures (Fig. 1E and F). Because the nucleoids in cells with abnormal wall morphologies had a highly variable appearance, we did not attempt to quantify them in our scoring. Anucleate cells in the *recA* strain were rare (Table 1). In other experiments in which a larger number of cells were observed, the anucleate frequency of two different *recA* alleles was 0.16% (4 anucleate cells out of 2,500 cells scored). This is notable because a hallmark of *E. coli recA* strains is their high frequency of anucleate cells (~10%) (38). Anucleate cells in *E. coli recA* strains are thought to result from "*rec*-less" degradation of chromosomal DNA mediated by RecD (29).

Two major homologous recombination pathways (RecA dependent) are responsible for processing of stalled replication forks in *E. coli*: the *recBCD* pathway and the *recF* pathway (5). The RecD component of RecBCD has been identified as the nuclease responsible for degradation of chromosomes in *recA* cells of *E. coli* (20). The counterparts for *E. coli recBCD* and *recF* in *B. subtilis* are *addAB* and *recF* (9). Like the *recA* strain, both the *addB* and *recF* strains showed a low frequency of nucleoid partitioning defects. Neither strain, however, showed substantial signs of the cell wall irregularities noted in *recA* and *recA* combination mutants (Table 1). Consistent with previous experiments, *ripX* and *ripX recA* strains displayed strong evi-

dence of nucleoid partitioning defects. We also note that the frequency of cell wall defects observed with the *ripX recA* strain was more than triple that seen with the *recA* strain, suggesting that there is an additive penalty for carrying both mutations (Table 1).

Viability of *B. subtilis rec* mutants during exponential-phase growth. The generation time of the *recA* strain in LB medium was significantly longer than that of the parent strain (*recA*, 31 min; parent, 22 min). The plating efficiency of an exponentially growing *B. subtilis recA* strain was about 12% of that seen in the parent strain (Table 1). This score is in agreement with previous results for a *recA* mutant grown at 30°C (14) and is particularly striking given the mild nucleoid phenotype we have observed. These data are consistent with the concept that *recA* cells suffer from an inability to repair stalled DNA replication forks. The *B. subtilis recA* viability as indicated by plating efficiency is less than the 50% viability score reported for *E. coli recA* mutants (4). The viabilities of the *addB* and *recF* mutants were about 50 and 60%, respectively (Table 1). The *addB recF* double mutant was approximately 30% viable, reinforcing the concept that both recombination paths are utilized during exponential growth. The coparticipation of the *B. subtilis* AddAB and RecF recombination pathways has previously been shown in transformation and transduction assays (1). In addition, the double-mutant data suggest that a small amount of recombination repair can be mediated independent of AddB and RecF. We note that the presence of a *ripX* mutation (which impairs chromosome dimer resolution) in a *recA* strain lowered the plating efficiency twofold (from 12% to 5.6%). This result supports our observation that *ripX* mutations are not silent in a *recA* derivative of strain BR151 (26).

Although the frequency of anucleate cells in *B. subtilis* was substantially lower than that seen in *E. coli*, the 12% viability of the *B. subtilis recA* mutant suggests that most of the nucleated *recA* population is nonviable. Also, the sum of the nucleoid defects in *B. subtilis recA* cells, recorded here as 6%, presumably underreports the extent of nucleoid defects in a popula-
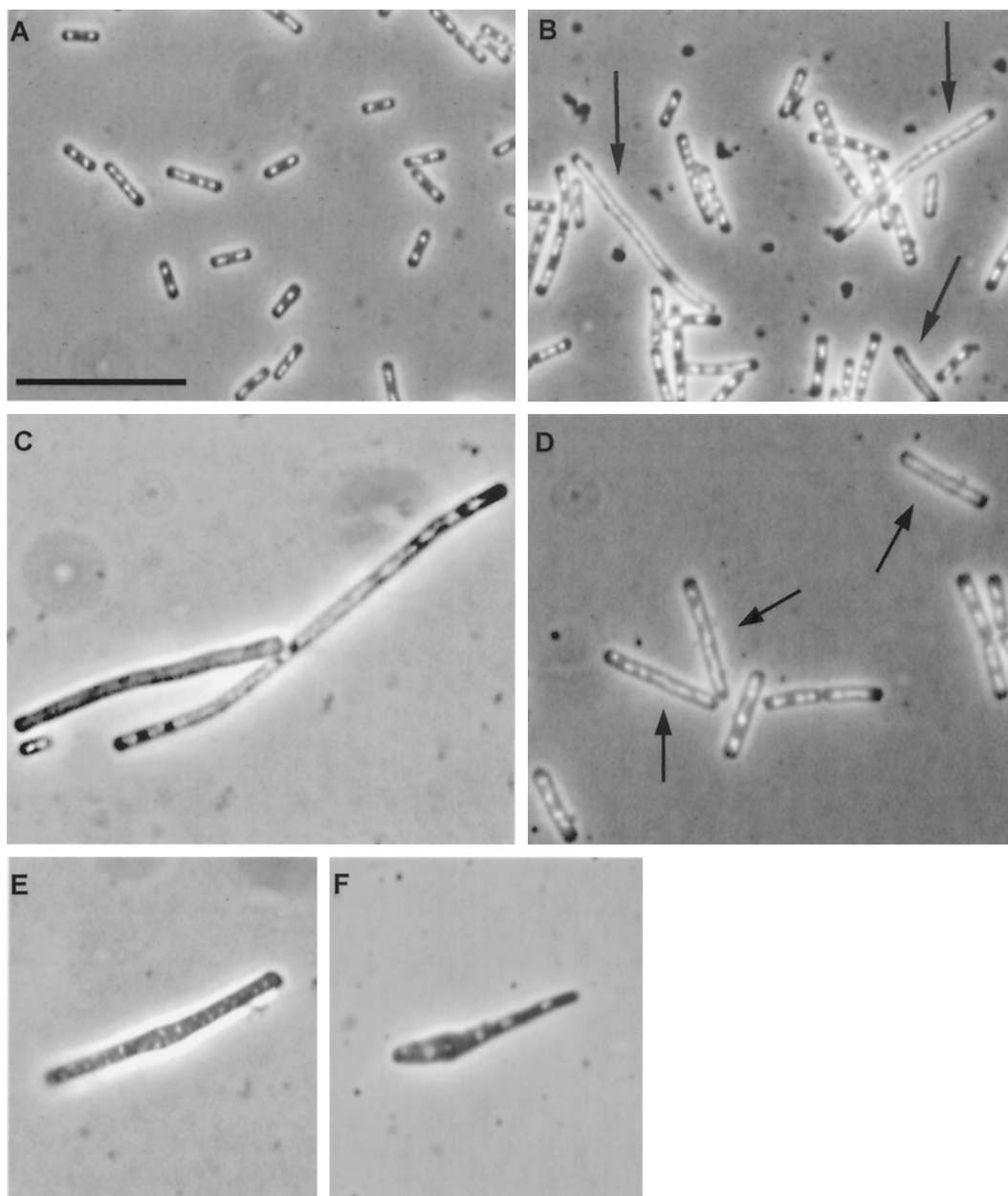
FIG. 1. Micrographs of *B. subtilis* strains treated with DAPI to visualize nucleoids. (A) Parent strain BR151 grown to stationary phase ($T_{1.5}$) in LB medium. (B) *recA* mutant SL7360 grown to stationary phase ($T_{1.5}$) in LB medium. (C) *recA* mutant SL7360 grown to stationary phase ($T_{1.5}$) in sporulation medium MSSM. (D) Parent strain BR151 grown in LB medium with MMC (20 ng/ml) added at mid-exponential phase; photo was taken 1.5 h after MMC addition. (E) Example of cell wall defect in *recA* mutant SL7360 during the exponential phase. (F) Example of cell wall defect in *recA ripX* mutant SL7370 during the exponential phase. Arrows point to elongated nucleoids. The scale bar in panel A is 10 μm and applies to all panels.

tion. Based on these considerations, we speculate that lethal nucleoid damage indeed occurs in *B. subtilis*, but degradation is not as rapid or complete as it is in *E. coli*.

Cell division and nucleoid phenotypes of stationary-phase *recA* cells. After the end of exponential growth, *recA* mutants developed a subpopulation of filamented cells with elongated nucleoids (Fig. 1). These unusual cells have been measured as a separate class apart from normal cells to highlight the differences between the two cell types in the *recA* stationary-phase population. In Table 2, we show that the peak represen-

tation of the aberrant subpopulation is about 17% (a similar frequency was observed for a *recA4* point mutant strain [data not shown]). The average nucleoid length in the aberrant subpopulation class was found to be two- to fourfold greater than that in the normal-appearing *recA* cells and in the parent strain. Also, the average cell length of the aberrant subpopulation was two- to threefold larger than that of unaffected *recA* cells and that of the parent strain (Table 2 and Fig. 1). We observed no signs of septum formation in the areas occupied by these distended *recA* nucleoids, either by phase-contrast

TABLE 2. Cell and nucleoid measurements in strains during the transition from exponential growth to the stationary phase

Time (min)[a]	Result for strain (relevant genotype):							
	BR151 (parent)			SL7360 (recA::neo)				
					Normal class		Elongated class	
	Frequency of elongation (%)[b]	Avg nucleoid length (μm)[c]	Avg cell length (μm)[d]	Frequency of elongation (%)[b]	Avg nucleoid length (μm)[c]	Avg cell length (μm)[d]	Avg nucleoid length (μm)[c]	Avg cell length in μm[e]
−20	0.7	0.93	4.9	3.8	0.83	5.5	1.3	11.4 (2)
0	0.2	0.84	4.9	7.5	0.76	6.3	1.6	9.1 (5)
20	0.5	0.88	4.4	10.5	0.89	5.6	1.5	10.2 (6)
40	0.2	0.8	4.3	12.9	0.82	5.3	3	9.8 (6)
60	1.3	0.87	4.1	14.3	0.7	3.6	2.8	9.5 (7)
80	0.7	0.77	3.1	12.3	0.67	3.3	2.4	7.5 (6)
100	0.2	0.73	2.6	16.8	0.7	3.2	2.8	6.9 (7)

[a] Time relative to the end of exponential growth.
[b] Elongated in this experiment refers to nucleoids whose length was >50% of the cell length.
[c] A minimum of 150 nucleoids were measured per time point in a minimum of 15 random fields of view.
[d] At least 45 cells were measured per time point in a minimum of 15 random fields of view.
[e] The number of cells measured in the SL7360 elongated class is indicated in parentheses.

microscopy or by fluorescent microscopy employing the vital membrane stain FM4–64 (22) (Fig. 1) (data not shown).

Based on the role of RecA in restoration of stalled replication forks, it is plausible that some or all of the cells in stationary-phase cultures of the *B. subtilis recA* strains that contained elongated nucleoids had accumulated DNA damage that prevented replication fork progression. This supposition is strengthened by our observation that treatment of *B. subtilis* RecA⁺ cells with MMC resulted in the development of filamented cells containing elongated nucleoids, a phenotype similar to that seen in *recA* stationary-phase cells (compare Fig. 1B and D). Additionally, *recF* and *addB* mutants showed the same distinctively elongated cells and nucleoids during stationary phase, although not as frequently (50 and 10% relative to the *recA* mutant [data not shown]). We discuss the growth-phase-specific nature of the aberrant subpopulation in *B. subtilis recA* cells later.

The reduced frequency of aberrant nucleoids in *addB* cells was unexpected. A possible explanation is that in the absence of RecA or RecF, unprocessed DNA substrates subsequently become targets for the exonuclease activity of AddAB. This combination of DNA degradation without repair might lead to the observed stationary-phase nucleoid phenotype in *recA* and *recF* cells. If this proposed scheme is correct, *addB* mutants would not be expected to present similar frequencies of the aberrant nucleoid phenotype to those seen in *recA* and *recF*

mutants, because chromosomal DNA would not be efficiently degraded in the absence of a fully functional AddAB complex.

In an *E. coli recA* mutant (W3110), we observed no comparable signs of nucleoid elongation during the transition from exponential growth into the stationary phase (data not shown). Furthermore, the amount of cell filamentation in *E. coli recA* cells actually decreased during this time (exponential, ~13%; stationary, ~4%). It is also worth noting in this context that even when treated with MMC, *E. coli recA* cells continued growing and dividing at stages where plating yielded no CFU (13). Therefore, a second distinction can now be made between *E. coli recA* and *B. subtilis recA* cells. Whereas chromosome degradation is extensive and cell division occurs frequently in *E. coli recA* cells, the more stable nucleoids appear to persist as barriers to septation during the transition into the stationary phase in *B. subtilis recA* cells (31, 35). A similar absence of septation in cells harboring aberrant nucleoids has been seen in *B. subtilis ripX* mutants (26).

Why should *E. coli* and *B. subtilis* nucleoids appear so different upon entry into stationary phase? *E. coli* H-NS and integration host factor (IHF) are DNA-binding proteins involved in maintaining nucleoid structure and have been shown to be transcriptional regulators of gene expression. The IHF protein is up-regulated upon entry into the stationary phase (7). While several groups have shown substantial H-NS up-regulation at the stationary phase (6, 30, 32), another study

TABLE 3. Cell density and sporulation frequencies of parent, *recA*, and *recF* strains

Strain (relevant genotype)	Absolute no. of CFU[a]		Sporulation (%)[b]	Frequency relative to parent		
	No heat	Heat		Sporulation	Viable count	Heat survival
BR151 (parent)	8.3×10^7	5.5×10^7	66	1.0	1.0	1.0
SL7360 (recA::neo)	8.1×10^6	3.6×10^6	45	0.68	0.097	0.067
SL7609 (recF::spc)	5.9×10^7	2.9×10^7	49	0.74	0.71	0.52

[a] Numbers refer to the number of CFU normalized to bacterial dry weight (in milligrams) observed on LB agar. See text for details.
[b] Sporulation was assessed 21 h after the cessation of exponential growth. The percentages shown here were within 2% of those obtained by phase-contrast microscopic examination just prior to plating. Heat treatment was for 20 min at 80°C.

TABLE 4. Prespore nucleoid condensation at different times after the start of sporulation

Strain (relevant genotype)	% of condensed nucleoids in prespore compartment[a]				
	T_2^b	T_3	T_4	T_5	T_6
BR151 (parent)	2.5	27.2	43	38.8	22.5
SL7360 (recA::neo)	0.2	0.3	1.6	11.7	11.4
SL7609 (recF::spc)	1.1	14.4	27.8	32.4	22.3

[a] The results shown are combined data from two independent experiments. A minimum of 500 cells were scored per time point per strain.

[b] T, time in hours after the end of exponential growth.

showed little growth phase variation in H-NS levels (10). The *B. subtilis* DNA-binding proteins with the strongest similarity to these *E. coli* proteins are HBsu (IHF) and Smc (H-NS) (2, 8, 17, 18). Interestingly, *B. subtilis* transcription of *hbs* (encoding HBsu) and the Smc level are both reduced in stationary phase (8, 11), although the HBsu/DNA ratio in spores is similar to that in vegetative cells (24). It is possible, therefore, that *E. coli* has evolved a more comprehensive system of stationary-phase DNA compaction and protection than *B. subtilis* (with the exception of the highly protective stationary-phase sporulation program).

recA phenotypes during sporulation. As was the case in LB medium, only about 10% of sporulating *recA* cells were viable (Table 3). The same morphological phenotypes found in *recA* cells making the transition from the exponential phase into the stationary phase under nonsporulation conditions were also seen in postexponential *recA* cells in the sporulation-inducing medium MSSM (Fig. 1C). The elongated nucleoid structures seen in postexponential *recA* cells were distinguishable from sporulation-dependent axial filaments that form during stage I of sporulation in *B. subtilis* (3) as follows. (i) Axial filaments were not formed by the parent strain in LB medium. (ii) The cell and nucleoid lengths were both noticeably larger in affected *recA* cells than those of the parent strain in sporulation
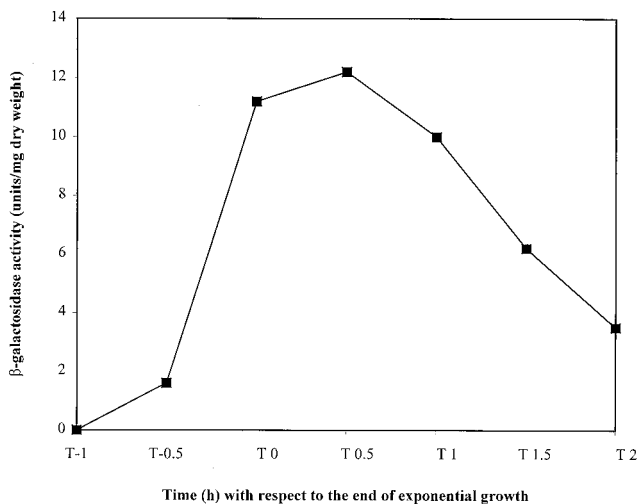

FIG. 2. *recA-lacZ* activity during the transition into the stationary phase. Solid diamonds, SL7937 (*recA-lacZ*). The endogenous activity of the parent strain BR151 has been subtracted from each time point. Results are the average of two experiments.

medium at stage I of development. (iii) Unlike stage I sporulation axial filaments, whose appearance is temporary, the phenotypes associated with *recA* cells that became apparent at the end of exponential growth were maintained for at least 7 h (data not shown).

In addition to the cell division and nucleoid irregularities seen early in the sporulation program, we have observed two separate and unfavorable consequences for sporulating *recA* mutants. First, the timing of prespore nucleoid condensation (27) is delayed by ~2 h and occurs at a lower frequency than that of the parent strain (Table 4). Second, *recA* strains sporulate less frequently than their parent strain (Table 3) (26); the reduced sporulation frequency is distinct from the altered resistance properties of *recA* spores (28). The impairment in prespore condensation and diminished frequency of sporulation are likely related to the aberrant morphologies discussed previously. Regardless of the cause, it should be remembered that the reduced sporulation frequency in *recA* mutants is superimposed on a severely reduced plating efficiency.

Induction of recA during the stationary phase. Using a *recA-lacZ* transcriptional fusion, we have shown that at approximately the same time *recA* cells begin developing overt cell division and nucleoid irregularities, transcription of the *recA* gene in BR151 was induced at the end of exponential gowth in LB medium (Fig. 2). (In other experiments, a low, basal level of transcription was observed during exponential growth.) This postexponential induction is in agreement with previous observations of *recA* transcription in *B. subtilis* in LB medium (23) and in competence medium (15, 23). RecA is capable of positively regulating gene expression (33, 36). Therefore, we reasoned that the elongated nucleoids in a subpopulation of cells in the stationary phase (Fig. 1B) might be the result of gene silencing in the absence of the normally induced RecA. For example, *B. subtilis mecA* mutants release *comK* transcription and the transcription of other ComK-regulated genes, such as *comC, -D, -E,* and *-G,* from medium dependence and have been reported to have cell and nucleoid abnormalities similar to those we have seen in stationary-phase *recA* cells (12). Based on these similarities, we speculated that RecA might be involved in the positive regulation of *mecA* during the stationary phase in the non-competence-inducing medium used here. Therefore, we evaluated the expression of *comK* and *comG* in our parent and *recA* strains during their transitions into the stationary phase. Neither the *comK-lacZ* nor the *comG-lacZ* reporter activity was enhanced in the absence of RecA (data not shown). These data indicate that RecA does not exert a positive regulatory effect on *mecA* or other genes responsible for regulating *comK* during the stationary phase in LB medium.

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