JOURNAL OF BACTERIOLOGY, Mar. 2000, p. 1650–1658 Vol. 182, No. 6
0021-9193/00/$04.00+0

Penicillin-Binding Protein-Related Factor A Is Required for Proper Chromosome Segregation in *Bacillus subtilis*

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Received 11 October 1999/Accepted 17 December 1999

Previous work has shown that the *ponA* gene, encoding penicillin-binding protein 1 (PBP1), is in a two-gene operon with *prfA* (PBP-related factor A) (also called *recU*), which encodes a putative 206-residue basic protein (pI = 10.1) with no significant sequence homology to proteins with known functions. Inactivation of *prfA* results in cells that grow slower and vary significantly in length relative to wild-type cells. We now show that *prfA* mutant cells have a defect in chromosome segregation resulting in the production of ~0.9 to 3% anucleate cells in *prfA* cultures grown at 30 or 37°C in rich medium and that the lack of PrfA exacerbates the chromosome segregation defect in *smc* and *spoOJ* mutant cells. In addition, overexpression of *prfA* was found to be toxic for and cause nucleoid condensation in *Escherichia coli*.

Penicillin-binding proteins (PBPs), which catalyze the polymerization and cross-linking of bacterial peptidoglycan, can be divided into three classes based on their amino acid sequence: the low-molecular-weight PBPs and the high-molecular-weight (HMW) class A and class B PBPs (8). The HMW class B PBPs are monofunctional transpeptidases, some of which have essential functions in septation and maintenance of cell shape (8), while the HMW class A PBPs have both transglycosylase and transpeptidase activities (14, 42) and appear to be somewhat functionally redundant (17, 34). The *Bacillus subtilis ponA* gene, coding for the HMW class A PBP1, is transcribed predominantly during log-phase growth (34). Previous work with *B. subtilis* mutants lacking one or several of the three known HMW class A PBPs (PBP1, PBP2c, and PBP4) showed that (i) lack of PBP1 results in slower growth, increased cell length, and decreased cell diameter and (ii) PBP1 is functionally more important than PBP2c and PBP4 (34). It was also recently demonstrated that PBP1 localizes to cell division sites and plays an important role in the formation of the peptidoglycan division septum in vegetative cells of *B. subtilis* (30).

The *ponA* gene is part of a two-gene operon that also includes *prfA* (PBP-related factor A [note that in the *B. subtilis* genome database, *prfA* is called *recU*]), which is located immediately upstream of and cotranscribed with *ponA* (33). *prfA* codes for a putative 206 residue, basic protein (pI of 10.1), which has no significant sequence homology to proteins with known functions. However, DNA sequencing has indicated that genes encoding similar proteins are present in a large number of gram-positive bacteria: a BLAST search (2) of *prfA* against completed and unfinished microbial genomes (preliminary sequence data were obtained from The Institute of Genomic Research Website at http://www.tigr.org) produced sequences with significant homology from 10 different gram-positive organisms, while no *prfA* homologs were detected in gram-negative bacteria by this analysis. The former organisms include *Enterococcus faecalis* (54% identity in a 192-amino-acid overlap), *Staphylococcus aureus* (58% identity in a 167-amino-acid overlap), *Streptococcus pyogenes* (51% identity in a 195-amino-acid overlap), *S. pneumoniae* (49% identity in a 124-amino-acid overlap), *Deinococcus radiodurans* (49% identity in a 124-amino-acid overlap), and *Mycoplasma genitalium* (32% identity in a 187-amino-acid overlap). For at least two of these species, namely, *S. pneumoniae* and *S. aureus*, the *prfA* genes are also located upstream of *ponA* homologs (25, 32). Although the *prfA* gene product has not yet been identified in *B. subtilis*, it is known that inactivation of *prfA* results in cells that grow ~50% more slowly than do wild-type cells and vary significantly in cell length. This phenotype is exacerbated greatly by the additional loss of PBP1 but not by the loss of either PBP2c or PBP4 (34). Finally, it has been shown that a mutation in *prfA* (*recU*::*cat*) renders cells more sensitive to DNA-damaging agents and decreases the efficiency of transformation, suggesting a possible role for *prfA* in DNA repair and homologous recombination (7). Given that PBP1 plays an important role in cell division in *B. subtilis* (30) and that a *prfA* mutation has a clear phenotype (34), we have investigated the function of *prfA* in detail. Our results indicate that *prfA* is required for proper chromosome segregation in *B. subtilis*.

MATERIALS AND METHODS

Plasmids and bacterial strains. The plasmids and bacterial strains used in this study are listed in Tables 1 and 2, respectively. Note that strains PS2061 (Δ*prfA*::*spc*) and PS2123 (Δ*prfA*) have deletions removing ~60 and ~42% of the *prfA* coding region, respectively, and are therefore almost certainly *prfA* null mutants (33; D. L. Popham and P. Setlow, unpublished results).

Growth of *B. subtilis*. *B. subtilis* strains were grown overnight at 30°C on 2× SG (18) or Luria-Bertani (LB) (10 g of tryptone per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter, 1 mM NaOH) agar plates with or without appropriate antibiotics, inoculated into 2× YT medium (16 g of tryptone per liter, 10 g of yeast extract per liter, 5 g of NaCl per liter) or 1× Penassay broth (PAB) (Difco), and grown at 30 or 37°C. In some cases, 1% (wt/vol) xylose was included in the media. Growth rates reported are those for cells in log-phase growth.

PCR and cloning procedures. To express *prfA* in *B. subtilis* from a xylose-inducible promoter, the putative ribosome-binding site and coding region of *prfA* (from bp 651 to 1300) (33) was amplified by PCR using primers prfA-SphI (5′-GCATGCGTCATGATTAGTTTAATAAGG-3′ [underlined nucleotides denote an *Sph*I site]) and prfA-B/S (5′-GGATCCTCAACTAGTACCTTTCGC ACCAGATGATGG-3′ [underlined nucleotides denote *Bam*HI and *Spe*I sites; note that the *Spe*I site results in the addition of two extra amino acid residues at the C terminus of PrfA]) and chromosomal DNA from wild-type *B. subtilis* (strain PS832) as a template. The PCR product (670 bp) was ligated into pCR 2.1 to generate plasmid pLP78, and the insert was sequenced, removed by digestion with *Bam*HI and *Sph*I, and ligated into pRDC19 digested with the same enzymes to generate plasmid pLP79, which was used to transform *B. subtilis* (Table 2).

To express *prfA* in *Escherichia coli*, the coding region of *prfA* (from bp 683 to 1300) (33) was amplified by PCR using primers prfA-Nco (5′-CCATG**GCT**AT

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TABLE 1. Plasmids used in this study

Plasmid	Characteristics or construction	Source or reference
pCR 2.1	*E. coli* PCR cloning vector; *bla kan*	Invitrogen
pET9d	*E. coli* protein expression vector; P_{T7}-*kan*	Novagen
pRDC19	*B. subtilis thrC* integration vector; *Pxyl xylR erm spc bla*	F. Arigoni
pNG7	*B. subtilis soj/spoOJ* integration vector; *bla spc*	13
pLP75	pCR2.1 plus 629-bp PCR product using prfA-NcoI and prfA-BamHI	This work
pLP76	pET9d plus 629-bp *Nco*I-*Bam*HI fragment from pLP75	This work
pLP78	pCR2.1 + 670-bp PCR product using prfA-SphI and prfA-B/S	This work
pLP79	pRDC19 + 668-bp *Sph*I-*Bam*HI fragment from pLP78	This work

TCGGTATCCTAATGGAAAAAC-3′ [underlined nucleotides denote an *Nco*I site; boldface nucleotides denote an extra alanine codon included to facilitate cloning]) and prfA-Bam (5′-GGATCCTCAACCTTTCGCACCAGATG-3′ [underlined nucleotides denote a *Bam*HI site]) and chromosomal DNA from wild-type *B. subtilis* (strain PS832) as a template. The PCR product (629 bp) was ligated into pCR 2.1 to generate plasmid pLP75, and the insert was sequenced, removed by digestion with *Nco*I and *Bam*HI, and ligated into pET9d digested with the same enzymes to generate plasmid pLP76, which was used to transform *E. coli* BL21(DE3)/pLysS (41). Transformants were selected on 2× YT agar plates containing kanamycin (50 μg/ml) and chloramphenicol (20 μg/ml), and one such transformant (strain LP77) was used for further studies.

Growth and induction of recombinant *E. coli*, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and N-terminal protein sequencing. Recombinant *E. coli* was grown at 37°C in 20 or 50 ml of 2× YT medium containing chloramphenicol (20 μg/ml) and kanamycin or ampicillin (both used at 50 μg/ml) to an optical density at 600 nm (OD_{600}) of ~0.5, protein expression induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultures were incubated at 37°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and N-terminal protein sequencing were carried out essentially as described previously (29, 31).

Fluorescence microscopy. To stain cell walls and nucleic acids of *B. subtilis* cells, 0.5 ml of a culture grown in 2× YT or PAB medium was fixed for 20 min at room temperature with 4.4% (wt/vol) paraformaldehyde–0.017% glutaraldehyde–28 mM sodium phosphate (pH 7), washed twice with phosphate-buffered saline (PBS) (8 g of NaCl per liter, 0.2 g of KCl per liter, 1.44 g of Na_2HPO_4 per liter, 0.24 g of KH_2PO_4 per liter [pH 7.4]), and resuspended in 100 μl of GTE (50 mM glucose, 20 mM Tris-HCl [pH 7.5], 10 mM EDTA). Lysozyme was added to 2 mg/ml, and the cells were immediately applied to poly-L-lysine-coated microscope slides. After 30 s, excess fluid was removed and bound cells were washed twice with PBS and allowed to dry completely. Following rehydration with PBS, the slides were blocked for 20 min at room temperature with 2% (wt/vol) bovine serum albumin in PBS and incubated for 1 h at room temperature in PBS containing 0.1% (wt/vol) bovine serum albumin, 2 μg of 4′,6′-diamino-2-phenylindole (DAPI; Sigma) per ml, and 2 μg of Oregon Green-conjugated wheat germ agglutinin (WGA; Molecular Probes) per ml or 10 μg of propidium iodide (PI) per ml. Finally, the cells were washed six times with PBS and the slides were mounted using the SlowFade antifade kit from Molecular Probes. They were visualized with a Zeiss Axiovert 100 fluorescence microscope equipped with a Plan-APOCHROMAT 100x or a Plan-NEOFLUAR 63x oil immersion lens (Zeiss) and a standard filter block for visualizing Oregon Green, DAPI, and PI. Images were captured with a cooled charged-coupled device camera using exposure times of 1 to 2 s for Oregon Green, 2 s for DAPI, and 0.5 to 2 sec for PI (due to variable staining efficiency, it was necessary to use different exposure times for different strains).

Fluorescence microscopy of recombinant *E. coli* cells induced for 0, 30, or 60 min was as for *B. subtilis* cells with the following changes: glutaraldehyde (0.11% [wt/vol]) was included in the fixation mix, lysozyme treatment and Oregon Green-conjugated WGA or PI were omitted, the lens used was a FLUAR 40x oil immersion lens (Zeiss), and the exposure time for DAPI was 400 ms. All images were transferred to a Power Macintosh computer and processed using Adobe Photoshop version 4.0.

Electron microscopy. Log-phase *B. subtilis* cells were fixed, processed, and analyzed by electron microscopy as described previously (37).

RESULTS

Chromosome segregation defect in *prfA* deletion mutants. Previously it was shown that cells of a *prfA* deletion mutant (Δ*prfA*::*spc*; strain PS2061) produce normal levels of PBP1 but grow more slowly and vary significantly in cell length compared to wild-type cells (33, 34). An identical phenotype was observed for strain PS2123, which contains an in-frame deletion of *prfA* (33). To analyze these *prfA* strains in more detail, log-phase cells grown at 30 or 37°C in PAB or 2× YT medium were fixed, stained with DAPI and PI or Oregon Green-conjugated WGA, and subjected to fluorescence microscopy to visualize DNA, septa, and/or cell walls. This analysis revealed that in addition to exhibiting an abnormal division pattern, cultures of both *prfA* strains contained significant numbers of anucleate cells, ranging from 0.9 to 3% of total cells depending on the precise conditions of the experiment (Table 3). An example of an anucleate cell is shown in Fig. 1B (arrow iv; the cell visible in the right-hand panel does not stain with DAPI) (see also Fig. 4B, arrows). Under similar conditions, cultures of wild-type cells (strain PS832) contained less than 0.1% anucleate cells (Table 3). Cultures of both *prfA* strains also contained a large proportion of cells (~34%) with abnormal nucleoid-staining patterns (Fig. 1A and B). The abnormal nucleoid-staining patterns observed include nucleoids that are asymmetrically positioned in the cell (Fig. 1A, arrow i; the arrow points to a large region of a cell lacking chromosomal DNA), nucleoids that appear bisected by or impinge upon the septum (Fig. 1A, arrow ii; note also the cell immediately above arrow ii), and large aggregates of nucleoids occupying an extensive part of the cell (Fig. 1B, arrow iii). Such abnormal nucleoid-staining patterns were essentially absent (i.e., were present at <1%) in wild-type cells analyzed in parallel (Fig. 1C) (see also Fig. 4A), indicating that these abnormal staining patterns were not a fixation artifact. Some of the abnormalities in the appearance of nucleoids in *prfA* cells were even more apparent upon electron microscopy (Fig. 2a to d). For example, nucleoids that were bisected by the septum were clearly visible in some dividing *prfA* cells (Fig. 2a and d, arrows), while other cells had aggregates of nucleoids that appeared stretched out (Fig. 2b and c). When wild-type cells were analyzed in parallel, no such defects were observed (Fig. 2e). These results there-

TABLE 2. Strains used in this study

Strain	Relevant genotype or characteristics	Reference or construction[a]
B. subtilis		
PS832	Wild type, Trp^+ revertant of strain 168	Laboratory stock
PS2061	Δ*prfA*::*spc*	33
PS2123	Δ*prfA* in frame	33
AG1468	*trpC2 pheA1* Δ*spoOJ*::*spc*	13
RB35	*trpC2 pheA1* Δ*smc*::*kan*	6
LP85	Δ*prfA*::*spc* Δ*thrC*::(*Pxyl-prfA xylR erm*)	pLP79→PS2061
LP88	Δ*prfA*::*spc* Δ*thrC*::(*Pxyl-prfA xylR erm*)	pRDC19→PS2061
LP89	Δ*thrC*::(*Pxyl-prfA xylR erm*)	pLP79→PS832
LP99	Δ*smc*::*kan*	RB35→PS832
LP101	Δ*prfA*::*spc* Δ*thrC*::(*Pxyl-prfA xylR erm*) Δ*smc*::*kan*	RB35→LP85
LP102	Δ*spoOJ*::*spc*	AG1468→PS832
LP105	Δ*prfA* Δ*spoOJ*::*spc*	pNG7→LP102
E. coli		
PS1404	BL21(DE3)/pLysS	41
PS2599	BL21(DE3)/pLysS pET11a-*dacC*	31
PS2602	BL21(DE3)/pLysS pET11a	41
PS2692	BL21(DE3)/pLysS pET11a-*dacC-NC*	31
LP77	BL21(DE3)/pLysS pET9d-*prfA*	pLP76→PS1404

[a] *B. subtilis* was transformed as described previously (3), and transformants were selected on 2× SG agar plates at 37°C (all except RB35 transformants) or LB agar plates at 30°C (RB35 transformants) with appropriate antibiotics at the following concentrations: 100 μg of spectinomycin per ml, 10 μg of kanamycin per ml, or 0-5 μg of erythromycin per ml plus 12.5 μg of lincomycin per ml.

TABLE 3. Growth rates and anucleate cell production of DNA segregation mutants[a]

Strain (relevant genotype)	Doubling time (min) in:			% of anucleate cells (no. of cells counted) in:		
	PAB, 30°C	PAB+xyl,[b] 30°C	2× YT, 37°C	PAB, 30°C	PAB+xyl,[b] 30°C	2× YT, 37°C
PS832 (wild type)	35	35	20	0 (331)	ND	<0.1 (1633)
PS2061 (Δ*prfA*::*spc*)	53	52	42	2.5 (753)	ND	1.6 (1316)
PS2123 (Δ*prfA* in frame)	52	ND[c]	40	0.9 (449)	ND	3 (459)
LP85 (Δ*prfA*::*spc Pxyl-prfA*)	56	36	ND	0.8 (397)	0 (325)	ND
LP99 (Δ*smc*::*kan*)	99	ND	ND	13 (407)	ND	ND
LP101 (Δ*prfA*::*spc Pxyl-prfA* Δ*smc*::*kan*)	97	94	ND	24 (520)	15 (527)	ND
LP102 (Δ*spoOJ*::*spc*)	38	ND	25	<0.1 (1375)	ND	1 (391)
LP105 (Δ*prfA* Δ*spoOJ*::*spc*)	—[d]	—	38	—	—	8.2 (426)

[a] Cells were grown and their growth rate and percentage of anucleate cells were determined as described in Materials and Methods.
[b] PAB medium containing 1% (wt/vol) xylose.
[c] ND, not determined.
[d] —, cells did not grow under the indicated conditions (see the text for details).

fore suggest that the *prfA* mutants are defective in chromosome segregation.

Membrane and wall defects of a *prfA* deletion mutant. In our electron microscopy analysis, we also occasionally observed *prfA* mutant cells with abnormal morphology, like the tortuous cell shown in Fig. 2d, and we found that ~16% ($n = 32$) of the cells had abnormal clusters or defects in the cell wall (Fig. 2c, arrow) resembling those previously observed in cells lacking PBP1 (30). In some cross-sections of *prfA* cells (~19%; $n = 32$) abnormal membrane structures or swirls were observed across the diameter of the cell (Fig. 3); these membrane swirls seemed to be formed by inward growth of the membrane at potential division sites, because wall ingrowths could sometimes be seen at similar positions (Fig. 3a and c). Presumably, septum formation was initiated at these sites but failed to go to completion due to uncoupling of wall and membrane ingrowth. Collectively, these morphological defects could reflect an additional role for PrfA in membrane and/or cell wall synthesis, or they could be secondary effects due to defective chromosome segregation.

Complementation of the Δ*prfA*::*spc* mutation. To rule out the possibility that some or all of the effects of the Δ*prfA*::*spc* mutation were due to polar effects on the expression of the downstream *ponA* gene, we placed a copy of *prfA* fused to a xylose-inducible promoter (*Pxyl*) at the *thrC* locus of the Δ*prfA*::*spc* mutant strain to generate strain LP85. As a control, Δ*prfA*::*spc* cells transformed with vector alone were used (strain LP88). When these cells were grown in 2× YT or PAB medium supplemented with 1% xylose, the growth, morphology, and chromosome segregation defects associated with the Δ*prfA*::*spc* mutation were fully complemented in strain LP85 but not in strain LP88, suggesting that these defects were indeed caused by inactivation of *prfA* and not by a polar effect on the downstream *ponA* gene (Table 3 and data not shown). This is consistent with previous findings that the level of PBP1 is normal in strain PS2061 (33). In the absence of xylose, LP85 cells grew like *prfA* cells and had a similar but slightly less severe nucleoid segregation defect compared to *prfA* cells (Table 3 and data not shown). We therefore suspect that some PrfA protein (but less than the wild-type level) is present in strain LP85 in the absence of xylose.

A conditional *prfA smc* double mutant. The *smc* gene (for "structural maintenance of chromosomes") is required for chromosome structure and partitioning in *B. subtilis* and inactivation of *smc* produces cells with chromosome segregation defects similar to but more severe than those observed for *prfA* cells. For example, *smc* null mutant cells grown at 23 or 30°C contain about 10 to 15% anucleate cells compared to ~0.9 to 3% for *prfA* cells (Table 3) (6, 26). We attempted to generate a *prfA smc* double mutant by transforming PS2061 (Δ*prfA*::*spc*) cells with chromosomal DNA from strain RB35 (Δ*smc*::*kan*) and selecting transformants on LB plates supplemented with kanamycin at 30°C (strain RB35 is temperature sensitive for growth in rich medium [6]). Although we obtained a number of very small colonies after 2 days of incubation, detailed analysis of several of these transformants suggested that they most probably contained suppressor mutations (results not shown). To avoid the problem of potential suppressor mutations, we therefore generated a conditional *prfA smc* mutant by transforming strain LP85 (Δ*prfA*::*spc* Δ*thrC*::[*Pxyl-prfA xylR erm*]) cells with chromosomal DNA from strain RB35 (Δ*smc*::kan) and selecting transformants at 30°C on LB-kanamycin plates with or without 1% xylose. Interestingly, ~30-fold more colonies were produced when the transformants were selected on xylose-containing plates than on xylose-free plates (744 and 25 colonies on average, respectively, in two independent experiments), indicating that the combined effects of the *prfA* and *smc* mutations are detrimental to the cell. One of the transformants (strain LP101; Δ*prfA*::*spc* Δ*thrC*::[*Pxyl-prfA xylR erm*] Δ*smc*::*kan*) selected on a xylose-containing plate was chosen for further analysis. Analysis of LP101 cells grown at 30°C in PAB medium revealed no significant difference in the growth rate of cells grown with or without 1% xylose, which in both cases was similar to that of *smc* (LP99) cells (Table 3). However, LP101 cultures grown without xylose contained significantly more anucleate cells (~24%) than did cultures grown in the presence of xylose (~15% [Table 3]), suggesting that the lack of PrfA exacerbates the *smc* phenotype. Cultures of *smc* (LP99) cells grown under similar conditions contained ~13% anucleate cells (Table 3), which is consistent with previous reports (~10 to 15% anucleate cells [6, 26]). We also examined the LP101 cells grown to stationary phase in PAB medium with or without xylose. This analysis revealed the presence of extremely long chains of LP101 cells in the culture grown without xylose (Fig. 4F) while stationary-phase LP101 cells grown in the presence of 1% xylose looked like *smc* cells (Fig. 4E and data not shown).

Phenotype of a *prfA spoOJ* mutant. Another gene involved in DNA segregation in *B. subtilis* is *spoOJ*. SpoOJ is similar to the ParB family of plasmid-encoded partition proteins and is required for proper chromosome segregation during vegetative growth (13) and for correct chromosome positioning during sporulation (38). SpoOJ colocalizes with the origin region of the chromosome (9, 20, 22) and binds to specific sites located in the origin-proximal ~20% of the chromosome (21). Furthermore, the presence of one of these latter sites, called *parS*,

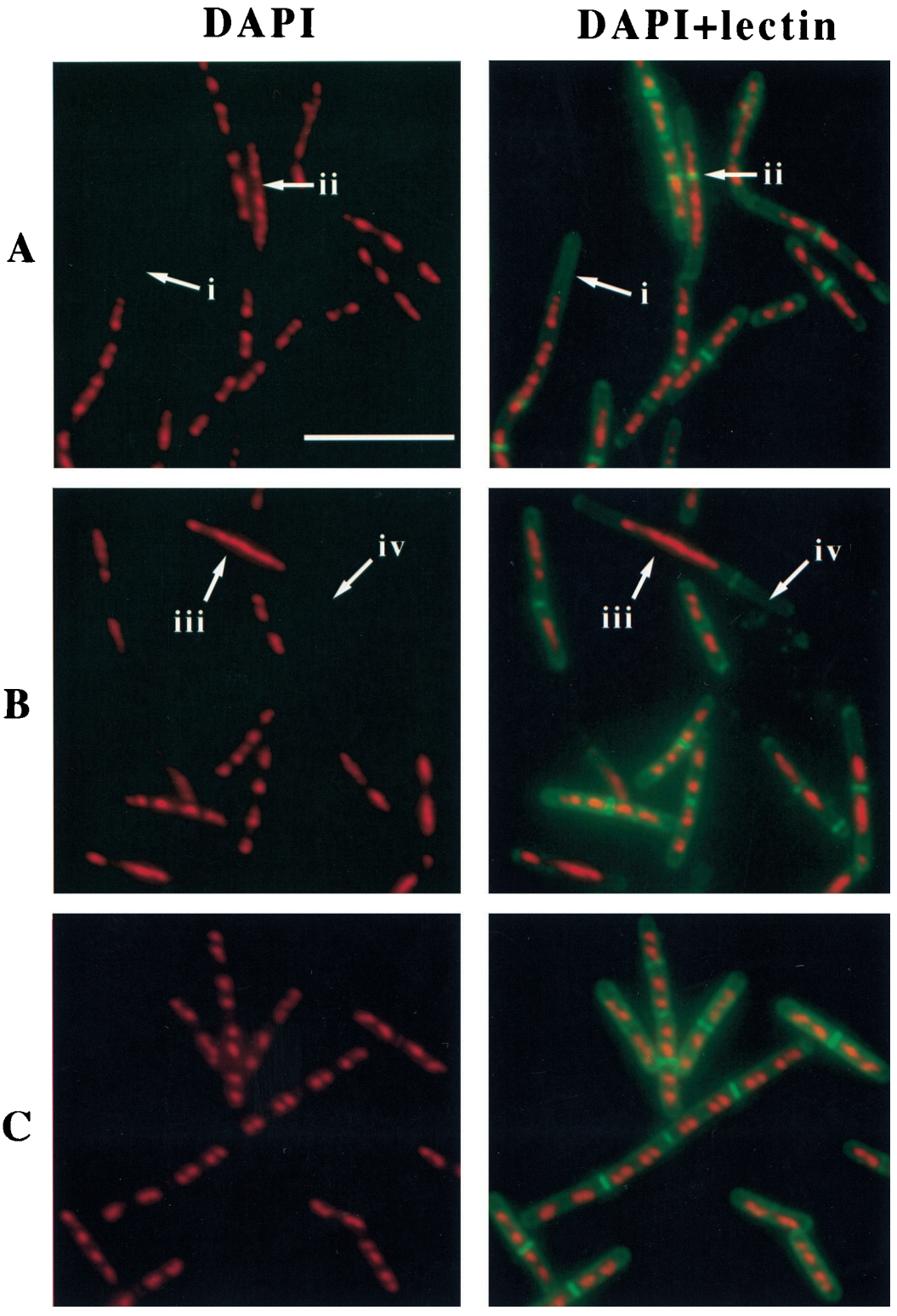

FIG. 1. Abnormal nucleoid staining of Δ*prfA*::*spc* (strain PS2061) mutant cells. Log-phase cells ($OD_{600} \approx 0.5$) of a Δ*prfA*::*spc* mutant (A and B) or a wild-type strain (C) grown at 37°C in 2× YT medium were fixed, stained with DAPI and Oregon Green-conjugated WGA (lectin), and analyzed by fluorescence microscopy to visualize nucleoids (pseudocolored red) and septa and cell walls (green). Arrows: (i) a cell with asymmetrically positioned nucleoids; (ii) a nucleoid that impinges upon the septum; (iii) a cell containing extended, aggregated nucleoids; (iv) an anucleate cell. Bar, 10 μm.

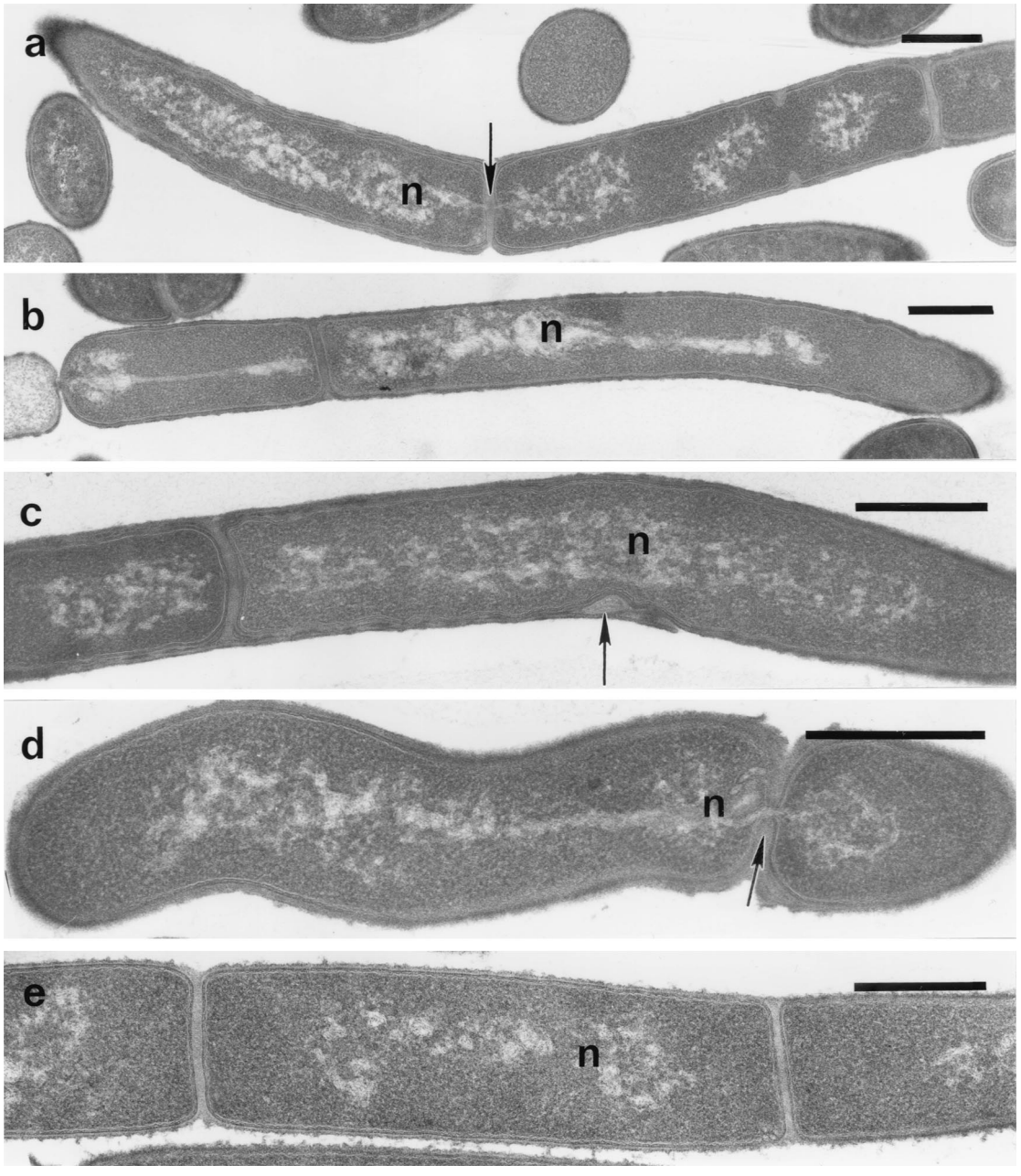

FIG. 2. Electron micrographs of cross-sectioned *prfA* (a to d) and wild-type (e) cells grown to log phase ($OD_{600} \approx 0.5$) at 37°C in 2× YT medium. The *prfA* strain used was PS2061 (Δ*prfA*::*spc*). Bars, 0.6 μm. Nucleoids (n) appear as light, fibrillar material in the cytoplasm. Arrows show nucleoids bisected by septa (a and d) and an abnormal cell wall cluster (c).

on an otherwise unstable plasmid stabilizes the plasmid in a SpoOJ-dependent manner, indicating that *parS* acts as a partitioning site (21).

To test for functional redundancy between PrfA and SpoOJ, we generated a *prfA spoOJ* double mutant by transformation of strain PS2123 (Δ*prfA* in frame) with linearized plasmid pNG7 to generate strain LP105 (Δ*prfA* Δ*spoOJ*::*spc*). This strain failed to grow at 30°C in PAB medium but grew like *prfA* cells in 2× YT medium at 37°C (Table 3). Although we have no clear explanation for this result, we note that *ponA* cells require increased levels of Mg^{2+} for growth and fail to grow in PAB medium due to its relatively low Mg^{2+} content (28). Perhaps strain LP105 has a similar requirement for increased Mg^{2+} levels. Fluorescence microscopy of log-phase *prfA spoOJ* cells grown in 2× YT medium at 37°C revealed that this mutant produced a much larger number of anucleate cells (~8.2%) than did either *prfA* or *spoOJ* single mutants (0.9 to 3 and 1%, respectively [Table 3]) and had defects in nucleoid appearance similar to those of *prfA* cells (compare Fig. 4B and D). This suggests that while a *prfA spoOJ* double mutant is viable, inactivation of *prfA* exacerbates the chromosome segregation defect of a *spoOJ* mutant.

Overexpression of *prfA* causes nucleoid condensation in *E. coli*. We next tried to overexpress *prfA* in *B. subtilis* by generating strain LP89 (Δ*thrC*::[*Pxyl-prfA xylR erm*]) and growing the cells in 2× YT medium supplemented with 1% xylose.

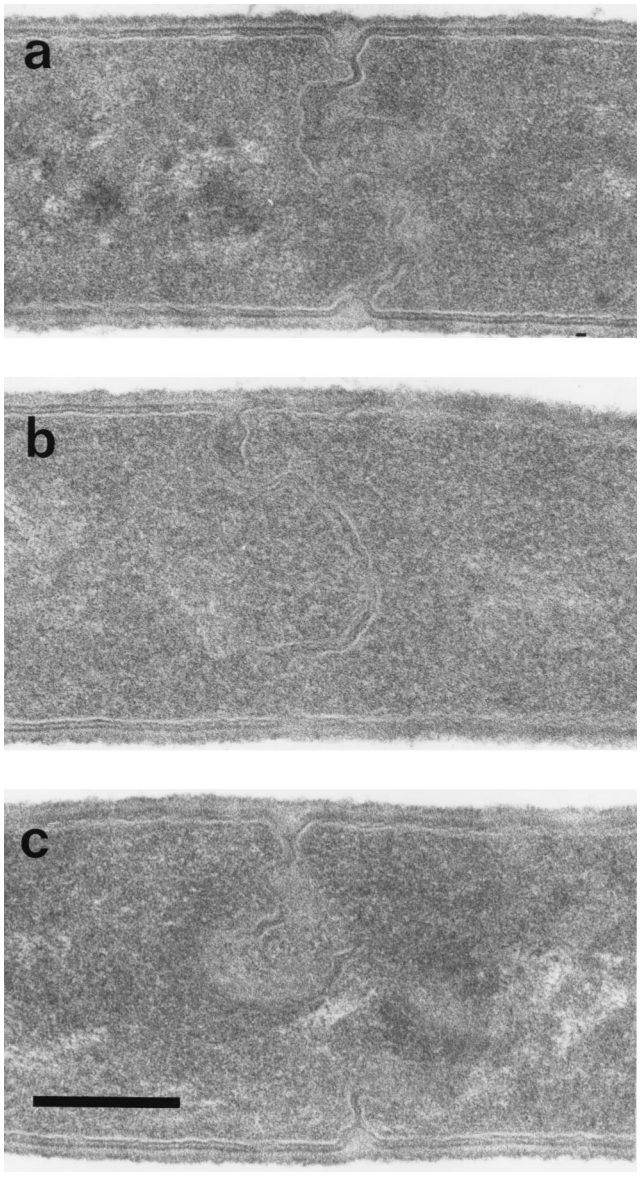

FIG. 3. Electron micrographs of cross-sectioned *prfA* (strain PS2061) cells showing abnormal membrane ingrowths or swirls across the diameter of the cell. Note the presence of wall ingrowths in the periphery of the cells shown in panels a and c. The cells were grown to an OD_{600} of ca. 0.5 at 37°C in 2× YT medium. Bar, 0.25 μm.

While LP89 cells grew normally under these conditions and were indistinguishable from wild-type cells, by SDS-PAGE analysis we could not detect any differences in protein profiles between xylose-induced LP89 and Δ*prfA*::*spc* cells (data not shown), suggesting that PrfA is not very abundant even in induced LP89 cells. We therefore cloned *prfA* in the expression vector pET9d and introduced it into *E. coli* BL21(DE3)/pLysS cells (41) to generate strain LP77. Induction of cells of strain LP77 with IPTG led to significant overproduction of a ~24.5-kDa protein, whose identity as PrfA was confirmed by N-terminal sequencing (data not shown). The overexpressed PrfA was found largely in inclusion bodies, although a significant amount appeared to be cytoplasmic and the protein was toxic for *E. coli* (data not shown).

The induced LP77 cells were analyzed by fluorescence microscopy after staining of nucleoids with DAPI. As controls we used induced cells of strain PS2602 carrying vector alone and induced cells of strain PS2599 producing the low-molecular weight PBP4a, which is toxic for *E. coli* when overexpressed (31). This analysis revealed a bright, punctuate nucleoid-staining pattern of cells expressing *prfA* (Fig. 5B), while the nucleoid-staining pattern of both control strains was dimmer and more diffuse (Fig. 5A and D). Cells with bright, punctuate nucleoids were also visible in a mixed culture of induced LP77 and PS2602 cells (Fig. 5C, arrows), indicating that the bright, punctuate staining of induced LP77 cells was not a microscopy artifact. Therefore, we conclude that overexpression of *prfA* causes nucleoid condensation in *E. coli*.

DISCUSSION

Following DNA replication, bacterial cells segregate daughter chromosomes into daughter cells with high fidelity, resulting in the presence of fewer than 0.03% anucleate cells in growing cultures of *E. coli* or *B. subtilis* (11, 13). The events involved in chromosome segregation and partitioning include resolution of chromosome dimers resulting from recombinational crossovers between sister chromosomes, decatenation of interlinked daughter chromosomes, and movement of daughter chromosomes away from each other (43). Recent evidence suggests that bacterial chromosome segregation is an active mitosis-like process and that the origin of replication (*oriC*) of the *E. coli* and *B. subtilis* chromosome has a specific orientation during the cell cycle (9, 10, 20, 44). Thus, in newborn cells *oriC* is oriented toward a cell pole; after replication of this region, one of the two origins moves rapidly toward the opposite pole of the cell while the termination region remains centrally located (10, 44).

A number of genes thought to be involved in the orientation and separation of daughter chromosomes have been characterized in *E. coli* and *B. subtilis* (reviewed in reference 43). These include *E. coli xerC* and *xerD* and their *B. subtilis* homologs *ripX* and *codV*, which code for recombinases involved in site-specific recombination leading to resolution of chromosome dimers (4, 5, 36). In addition, six so-called *par* genes involved in decatenation of interlinked daughter chromosomes have been characterized, as well as a number of genes thought to be involved in chromosome movement and partitioning (reviewed in reference 43). Among the latter group are the *muk*, *minD*, and *ftsK* genes of *E. coli* (12, 23, 49) and the *smc*, *spoOJ*, and *spoIIIE* genes of *B. subtilis* (6, 13, 26, 45). Finally, recent work by Lemon and Grossman (19) indicates that the process of DNA replication itself may contribute to the movement and separation of daughter chromosomes.

***prfA* is required for proper chromosome segregation.** In the present work we have shown for the first time that *prfA* is required for proper chromosome segregation in *B. subtilis*. Thus, cultures of *prfA* cells grown at 30 or 37°C in rich medium were found to contain ~0.9 to 3% anucleate cells and a significant proportion (~34%) of cells with abnormal nucleoid staining patterns (Fig. 1 and 4B and Table 3). By analysis of a conditional *prfA smc* mutant (strain LP101) and a *prfA spoOJ* mutant (strain LP105), we found that inactivation of *prfA* also exacerbates the *smc* and *spoOJ* chromosome segregation phenotypes. This suggests that *prfA* affects chromosome segregation via a pathway different from those used by *smc* and *spoOJ*.

How does PrfA affect chromosome segregation? An obvious question arising from this work is what exact role PrfA plays in chromosome segregation. A possible DNA-binding activity of PrfA is supported by the findings that PrfA is very basic (pI of 10.1) and that overexpression of *prfA* causes nucleoid conden-

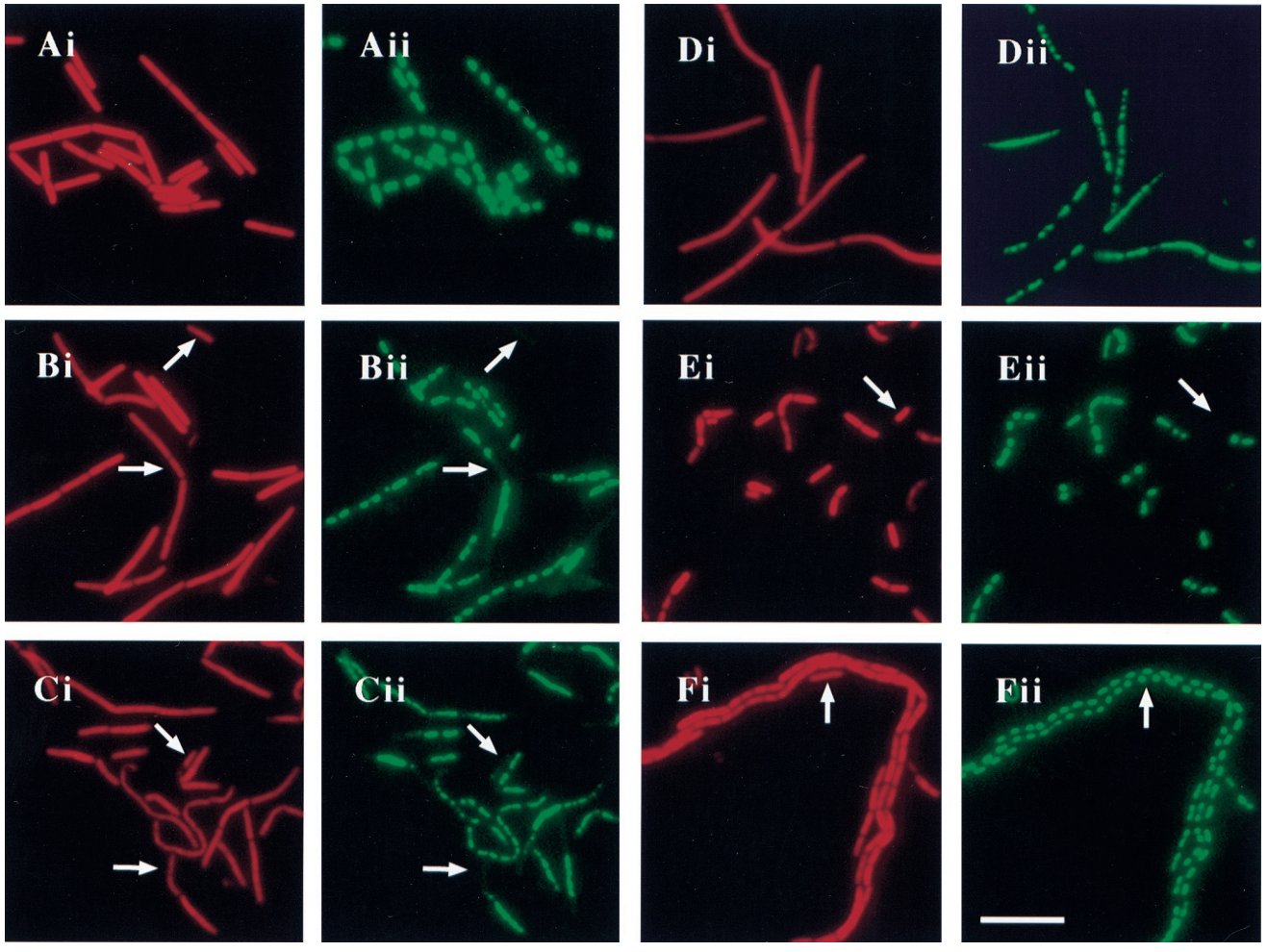

FIG. 4. Fluorescence micrographs of *prfA*, *smc*, and *spoOJ* strains grown in PAB medium at 30°C without (A, B, C, and F) or with (E) 1% xylose or in 2× YT at 37°C (D). The cells were fixed in log phase (A to D) or stationary phase (E and F) and stained with PI (Ai to Fi) to visualize the cytoplasm (red) and septa (dark lines between cells) and DAPI (Aii to Fii) to visualize nucleoids (pseudocolored green). (A) PS832 (wild type); (B) PS2061 (Δ*prfA*::*spc*); (C) LP99 (Δ*smc*::*kan*); (D) LP105 (Δ*prfA* Δ*spoOJ*::*spc*); (E and F) LP101 (Δ*prfA*::*spc* Δ*thrC*::[*Pxyl-prfA xylR erm*] Δ*smc*::*kan*). Cells of strain PS2123 (Δ*prfA*) appear identical to those of strain PS2061 (B); cells of strain LP102 (Δ*spoOJ*::*spc*) appear similar to the wild-type cells (A) Arrows indicate anucleate cells. Bar, 10 μm.

sation in *E. coli* (Fig. 5), although the latter finding could be a result of the basic nature of PrfA. If PrfA binds directly to DNA, the chromosome segregation defect seen in *prfA* cells could be due to impaired DNA replication, dimer resolution, decatenation, or chromosome movement and positioning. An effect of PrfA on DNA replication seems unlikely because the protein-to-DNA ratio in *prfA* cells is not significantly different from that of wild-type cells (L. B. Pedersen and P. Setlow, unpublished observations). Furthermore, in addition to anucleate cells, cells with increased DNA content were observed in *prfA* cultures (Fig. 1). Despite the ability of PrfA to induce DNA condensation when overproduced in *E. coli*, a general nonspecific DNA-condensing activity of PrfA in vivo also seems unlikely, given the presumed low abundance of this protein: PrfA has so far eluded detection by SDS-PAGE of extracts from wild-type *B. subtilis* cells, and PBP1, which is coexpressed with PrfA (33), is present in only 450 to 1,000 copies per cell (30).

Previous work has shown that a *prfA* mutant (*recU*::*cat*) is impaired in homologous recombination independently of other known *rec* genes (7). Since mutations in genes involved in homologous recombination are known to cause defects in chromosome segregation (15, 36, 50), it is possible that the chromosome segregation defect of *prfA* cells is due to a defect in homologous recombination. However, PrfA has no significant primary sequence homology to known recombinases, indicating that the effect of PrfA on homologous recombination may be indirect. Interestingly, the C-terminal domain of the *E. coli* FtsK protein, which is involved in chromosome segregation (23, 49), was recently shown to be required for resolution of chromosome dimers by site-specific recombination at *dif*, and it was suggested that FtsK could play a general role in preparing the nucleoid structure in a way that allows *dif* and the XerC/D recombinases to function properly (40). A XerC and XerD homologue, RipX, was recently identified in *B. subtilis*, and it was shown that *ripX* cells display chromosome segregation defects similar to those reported here for *prfA* cells (36). Perhaps PrfA is somehow involved in promoting RipX-mediated site-specific recombination in a manner similar to that proposed for FtsK.

Localization of PrfA? The C-terminal domain of FtsK is homologous to the SpoIIIE protein of *B. subtilis*. SpoIIIE is required for postseptational chromosome translocation in sporulating cells (45, 47) and in vegetative cells grown under

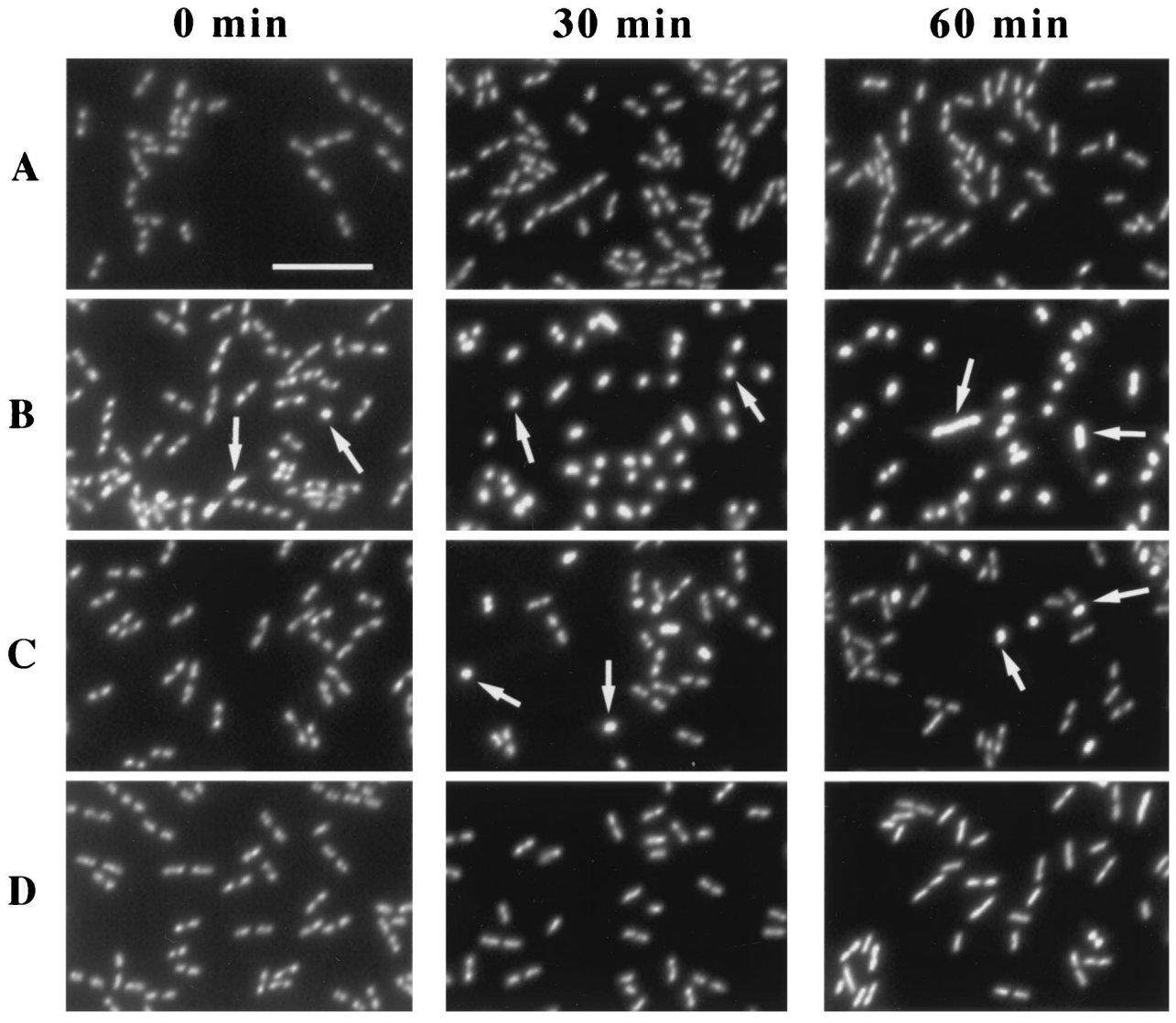

FIG. 5. Nucleoid condensation in *E. coli* expressing *prfA*. Cells were grown in 2× YT medium with appropriate antibiotics at 37°C and induced with IPTG for 0, 30, or 60 min, and the nucleoids were stained with DAPI and examined by fluorescence microscopy. (A) cells of strain PS2602 (pET11a); (B) cells of strain LP77 (pET9d-*prfA*); (C) mixture of PS2602 and LP77 cells; (D) cells of strain PS2599 (pET11a-*dacC*). Bar, 10 μm. Arrows show condensed nucleoids in cells expressing *prfA*.

conditions when normal nucleoid separation or septum positioning is perturbed (39). SpoIIIE localizes near the middle of the asymmetric division septum during sporulation and has been suggested to form a seal between the DNA and the leading edge of the division septum (46). Given that PBP1 localizes to the division septum and plays a role in its formation (30), it is tempting to speculate that PrfA is associated with the division septum and interacts with the chromosome in a manner similar to that proposed for FtsK and SpoIIIE. Determination of the cellular localization of PrfA would clearly help in clarifying these issues. Extensive attempts to localize PrfA by use of a PrfA-green fluorescent protein fusion have so far been unsuccessful (Pedersen and Setlow, unpublished), but efforts are under way to produce an antiserum against PrfA (D. L. Popham, personal communication).

Possible indirect effects of PrfA on chromosome segregation. Finally, it is possible that PrfA affects chromosome segregation indirectly by affecting septum formation, by recruiting other proteins to certain sites, or by acting as a chaperone for proteins involved in DNA-wall-membrane interactions. Although *prfA* cells vary greatly in length, suggesting a defect in septum placement (34), indirect evidence suggests that this phenotype may be a secondary effect of impaired chromosome segregation rather than vice versa. For example, studies with *E. coli parC* and *mukB* mutants have indicated that FtsZ ring formation and hence septation can be prevented to some extent by abnormally large or aberrant nucleoids (24, 48). Furthermore, mutations in *minD*, which is known to be involved in septum positioning (35), have no overt effect on chromosome segregation in *B. subtilis* (39), although such an effect has been reported for *E. coli* (1, 16, 27). Therefore we find it plausible that the impaired septum placement phenotype of *prfA* mutant cells (34) could be due to the presence of abnormally large or aberrant nucleoids in these cells.

Purification of PrfA, analysis of its biochemical activity, and determination of its three-dimensional structure may provide further insights into the structural and functional characteristics of this protein. This work is in progress.

ACKNOWLEDGMENTS

We thank David L. Popham and Alan Grossman for strains and plasmids, Fabrizio Arigoni for the gift of plasmid pRDC19, Arthur L. Hand for performing electron microscopy, and David Bishop-Bailey for help with fluorescence microscopy. Finally, we acknowledge The Institute for Genomic Research for making preliminary sequence data available on the Internet at http://www.tigr.org.

This work was supported by a grant from the National Institutes of Health to P.S. (GM19698) and a postdoctoral fellowship from the Danish Natural Science Research Council to L.B.P. (9601026).

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