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## SbcCD Regulation and Localization in *Escherichia coli*<sup>∇</sup>

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**The SbcCD complex and its homologues play important roles in DNA repair and in the maintenance of genome stability. In *Escherichia coli*, the in vitro functions of SbcCD have been well characterized, but its exact cellular role remains elusive. This work investigates the regulation of the *sbcDC* operon and the cellular localization of the SbcC and SbcD proteins. Transcription of the *sbcDC* operon is shown to be dependent on starvation and RpoS protein. Overexpressed SbcC protein forms foci that colocalize with the replication factory, while overexpressed SbcD protein is distributed through the cytoplasm.**

The maintenance of genome stability is crucial for all living organisms. External as well as internal factors can cause DNA lesions that are precursors to mutation or to DNA repair. In the gram-negative eubacterium *Escherichia coli*, the structural maintenance of chromosome proteins, such as the SbcCD complex, plays important roles in DNA repair and in the maintenance of genome stability. Mutations in the *sbcDC* operon facilitate the propagation of palindromes-containing replicons (6, 14) and are associated with the increased frequency of inversion between short inverted repeats (25). SbcCD is a complex that cleaves double-strand DNA, where SbcD comprises the nuclease center. SbcC consists of ATPase domains separated by long coiled coils and modulates the nuclease activity (7). Although several activities of the *E. coli* SbcCD complex have been biochemically characterized, its regulation and cellular localization have remained unknown.

In the gram-positive eubacterium *Bacillus subtilis*, localization of the PolC protein, a subunit of the DNA polymerase III, demonstrates that a replication factory through which the DNA template would travel resides in the middle of the cell (15). Localization of the *B. subtilis* SbcCD complex has been studied (16, 17). Native levels of the SbcC-yellow fluorescent protein (YFP) fusion protein are mostly cytoplasmic (16). However, in the presence of mitomycin C, a DNA cross-linking agent, SbcC-YFP seems to localize in the nucleoid and to colocalize with DnaX-CFP, another component of the replication factory. SbcD-GFP localized to the cytoplasm, independently of the presence of mitomycin C. Strikingly, mitomycin C induced the production of the SbcC-YFP and SbcD-GFP proteins. Another study showed the localization of overexpressed GFP-SbcC and GFP-SbcD fusion proteins (17). The GFP-SbcC fusion protein localized at a mid-cell spot in the nucleoid, displaying a pattern similar to that of subunits of the DNA polymerase III, such as DnaN or DnaX (17). Localization of overexpressed GFP-SbcD was cytoplasmic. These data would

suggest that in *B. subtilis*, SbcC but not SbcD may be associated with the replication factory.

In *E. coli*, the presence of a central fixed replication factory was established by microscopy, using the SeqA fusion protein (20). This protein binds newly formed hemimethylated DNA during replication and localizes at the replication fork. SeqA has been shown to be closely associated with the replication factory (9).

In *E. coli*, starvation, entry into the postexponential phase during growth, and other conditions of stress induce the RpoS sigma factor ( $\sigma^S$ ). The  $\sigma^S$  factor could be directly or indirectly involved in the regulation of 10% of *E. coli* genes (29). Regulation of the  $\sigma^S$  is complex and involves all stages of its production (10). Notably, the Hfq protein helps *rpoS* translation, whereas the ClpXP protease is responsible for degradation of the RpoS protein.

This work investigates SbcCD regulation and localization in *E. coli*. Expression of the *sbcDC* operon is shown to be dependent on starvation and RpoS. Localization of SbcCD can be detected only when proteins are overexpressed. Overexpressed SbcC is associated with the replication forks, while overexpressed SbcD is a cytoplasmic protein.

### MATERIALS AND METHODS

**Plasmids, bacterial strains, primers, and media.** Table 1 lists the plasmids, bacterial strains, and primers used. Bacteria were grown at 37°C with agitation in either LB (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, and 2 mM NaOH) or M9 medium (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 8.5 mM NaCl, and 2 mM NH<sub>4</sub>Cl, supplemented with 0.1 M CaCl<sub>2</sub>, 1 M MgSO<sub>4</sub>, and 0.2% glycerol). Antibiotics were used at the following concentrations: ampicillin, 100 mg/liter; chloramphenicol, 50 mg/liter; and kanamycin (Km), 50 mg/liter. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was used at 50  $\mu$ M. Arabinose was used at 0.001% (wt/vol).

**DNA techniques.** Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as described by Sambrook and collaborators (24). PCR was carried out as described by van Dijk et al. (27). Enzymes were from New England Biolabs or Roche Molecular Biochemicals.

The *E. coli* MG1655  $\Delta(P_{lac-lacZY}) \Delta sbcDC P_{sbcDC-lacZ-aph}$  reporter strain (DL1649; Fig. 1A) was constructed by replacement of the *sbcDC* operon with a DNA fragment containing the *lacZ* gene and the kanamycin resistance gene *aph*. Two amplified fragments flanking the *sbcDC* operon were ligated, after PCR-mediated coupling, into the chromosomal integration-and-excision plasmid pTOF24 (18). The upstream fragment of 450 nucleotides was amplified from *E. coli* MG1655 using primers sbcDC2-1 and sbcDC2-2. The downstream fragment of 466 nucleotides was amplified from *E. coli* MG1655 DNA using primers

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TABLE 1. Plasmids, bacterial strains, and primers

Plasmid, strain, or primer	Relevant properties <sup>b</sup>	Reference or source
<b>Plasmids</b>		
p18seqA	pLAU18 derivative plasmid carrying a SeqA-eYFP C-terminal fusion under the control of an arabinose-inducible promoter (P <sub>BAD</sub> ); Amp <sup>r</sup>	This work
p207NsbC	pDSW207 derivative carrying a GFP-SbcC N-terminal protein fusion; Amp <sup>r</sup>	This work
p207NsbCD	pDSW207 derivative carrying a GFP-SbcD N-terminal protein fusion; Amp <sup>r</sup>	This work
pDSW207	pTrc99A derivative containing a <i>gfp</i> -MCS region controlled by a downregulated <i>trc</i> promoter (mutation in -35 box); Amp <sup>r</sup>	30
pLAU18	pUC18 derivative permitting the construction of eYFP fusion proteins under the control of an arabinose-inducible promoter (P <sub>BAD</sub> ); carries the <i>araC</i> gene; Amp <sup>r</sup>	13
pRSET <sub>B</sub> DsRed	pRSET <sub>B</sub> derivative carrying the gene encoding the DsRed protein; Amp <sup>r</sup>	5
pseqAred	p18seqA derivative vector carrying the <i>dsRed</i> gene in place of <i>yfp</i> ; Amp <sup>r</sup>	This work
pseqAredKm	pseqAred derivative vector, where the <i>bla</i> gene is disrupted by the <i>aph</i> gene; Km <sup>r</sup>	This work
pTOF24	pSC101-based vector; <i>repA</i> (Ts) with a <i>sacB</i> gene conferring sucrose sensitivity and <i>aph</i> from pUC4K; Cm <sup>r</sup> , Km <sup>r</sup> , Ts, Suc <sup>s</sup>	18
pTOF30	Plasmid containing an FLK <sub>2</sub> cassette ( <i>lacZ aph</i> ); Amp <sup>r</sup> , Km <sup>r</sup>	18
pTOF24Δ <i>clpX</i>	pTOF24 derivative containing two fused PCR fragments corresponding to the beginning and the end of <i>clpX</i> , in place of the <i>aph</i> gene; Cm <sup>r</sup> , Ts, Suc <sup>s</sup>	This work
pTOFNGFP <i>sbcC</i>	pTOF24 derivative containing a GFP-SbcC N-terminal protein fusion and the ' <i>sbcD</i> ' sequence, permitting its integration at the locus in the <i>E. coli</i> chromosome; Cm <sup>r</sup> , Ts, Suc <sup>s</sup>	This work
pTOFNGFP <i>sbcD</i>	pTOF24 derivative containing a GFP-SbcD N-terminal protein fusion, P <sub><i>sbcDC</i></sub> , and part of the <i>phoB</i> gene sequence, permitting its integration at the locus in the <i>E. coli</i> chromosome; Cm <sup>r</sup> , Ts, Suc <sup>s</sup>	This work
pTOF <i>sbcDC</i>	pTOF24 derivative containing two fused PCR fragments corresponding to the beginning of the <i>sbcD</i> and the end of the <i>sbcC</i> genes, in place of the <i>aph</i> gene; Cm <sup>r</sup> , Ts, Suc <sup>s</sup>	This work
pTOF <i>sbcDCK2</i>	pTOF <i>sbcDC</i> derivative containing an FLK2 cassette from pTOF30; Cm <sup>r</sup> , Km <sup>r</sup> , Ts, Suc <sup>s</sup>	This work
<b>E. coli strains</b>		
BW27784	<i>lacI</i> <sup>q</sup> <i>rrnB3</i> Δ <i>lacZ</i> 4787 <i>hsdR514</i> DE( <i>araBAD</i> )567 DE( <i>rhaBAD</i> )568 DE( <i>araFGH</i> ) Φ( <i>ΔaraEp</i> P <sub><i>CPI8-araE</i></sub> )	11
DL1649	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) Δ <i>sbcDC</i> P <sub><i>sbcDC-lacZ-aph</i></sub>	This work
DL1718	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) Δ <i>sbcDC</i> <i>rpoS359::Tn10</i> P <sub><i>sbcDC-lacZ-aph</i></sub>	This work
DL1796	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) Δ <i>sbcDC</i> Δ <i>clpX</i> P <sub><i>sbcDC-lacZ-aph</i></sub>	This work
DL1913	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) Δ <i>clpX</i>	This work
DL2478	MG1655 <i>att::207NGFP-sbcC</i>	This work
DL2479	MG1655 <i>att::207NGFP-sbcD</i>	This work
DL2532	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) Δ <i>clpX</i> GFP- <i>sbcC</i>	This work
DL2533	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) GFP- <i>sbcC</i>	This work
DL2535	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) GFP- <i>sbcD</i>	This work
DL2739	BW27784 <i>att::207NGFP-sbcC</i>	This work
DL3117	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> ) <i>rpoS359::Tn10</i> GFP- <i>sbcC</i>	This work
EDCM367	MG1655 Δ(P <sub><i>lac</i></sub> - <i>lacZY</i> )	18
MG1655	F <sup>-</sup> λ <sup>-</sup> <i>ilvG rfb-50 rph-1</i>	2
RH90	MC4100 <i>rpoS359::Tn10</i>	12
<b>Primers<sup>a</sup></b>		
clpXflank F1	AAAAAGTCGACGCAGGGGGCAAAGGTAAC	This work
clpXflank F2	GGCTCAGGCAAATGGAAGACGTCGAAAAAGTGG	This work
clpXflank R1	CGACGCTCTCCATTGCGCTGACCCATCTTT	This work
clpXflank R2	AAAAACTGCAGCGCTTCCAGACACCGGATAG	This work
dsRED-XhoI	AAAAACTCGAGTTGGCTCCTCCGAGGACGTCATCAAG	This work
dsRED-HindIII	AAAAAAAGCTTTTATTCACCTGCAAACGTAC	This work
Km-ScaI-F	AAAAAAGTACTCCACGTTGTGTCTCAAAATC	This work
Km-ScaI-R	AAAAAAGTACTCCGTC AAGTCAGCGTAATG	This work
lacZ-chi-seq-R	TTT TTA TCG CCA ATC CAC ATC	This work
NtermGFP-SbcC1	AAAAACAATTGAACAACAACATGAAAATTCTCAGCCTGCG	This work
NtermGFP-SbcC2	AAAAAAAGCTTTTATTCACCTGCAAACGTAC	This work
NtermGFP-sbcC5	AAAAACTCGAGCCCCATTCCTGAGTTTGT	This work
NtermGFP-sbcC6	TTCTCCTTTACTCATGCTTCGTGTTCTCCGGC	This work
NtermGFP-sbcC7	ACACGAAGCATGAGTAAAGGAGAAGAAC	This work
NtermGFP-sbcC8	AAAAAGTCGACCTTTGTCCGGCGAGAATTTTG	This work
NtermGFP-SbcD1	AAAAAGAATTCAACAACAACATGCGCATCCTTCACACCTC	This work
NtermGFP-SbcD2	AAAAAAAGCTTTTCATGCTTCGTGTTCTCCGG	This work
NtermGFP-SbcD6	TTCTCCTTTACTCATAACCGGTTCCCTGGCG	This work
NtermGFP-SbcD7	GGAACCGTTATGAGTAAAGGAGAAGAAC	This work
SeqA_F	TTTCATGGATGAAAACGATTGAA	This work
SeqA_R	TTTTCGAGGATAGTTCGGCAAAC	This work
sbcC1-4	AAAAAGTCGACGTGAGTAGCGGCTGAAAAG	This work
sbcDC2-1	AAAAACTGCAGGGCCAGGGTTCATTGAGTT	This work
sbcDC2-2	CACACCGAGCGGCGCAGCAGCCAGTCAAGAAAAGC	This work
sbcDC2-3	TGGCTGCTCGCGCCGCTCGGTGTGATTAGCCACGTA	This work
sbcDCnd4-4	AAAAAGTCGACCCGCTGGCTGGTAATAATG	This work

<sup>a</sup> Primers are shown in a 5'-3' direction.

<sup>b</sup> Abbreviations: Cm<sup>r</sup>, chloramphenicol resistant; Km<sup>r</sup>, kanamycin resistant; Ts, replication of the plasmid is temperature sensitive; Suc<sup>s</sup>, sucrose sensitive. Restriction sites used for cloning are underlined, ATG start codons are double underlined, and sequences used for PCR-mediated coupling are indicated in bold type.

sbcDC2-3 and sbcC1-4. The 892-nucleotide-coupled fragment was cloned between the PstI and SalI sites of plasmid pTOF24, resulting in the kanamycin-sensitive plasmid pTOF*sbcDC*. The FLK2 cassette, carrying *lacZ* and *aph* genes, was isolated from the plasmid pTOF30 by restriction using the NotI enzyme and inserted into the cleaved plasmid pTOF*sbcDC*, resulting in pTOF*sbcDCK2*. This

temperature-sensitive plasmid was introduced into the *E. coli* MG1655 Δ(P<sub>*lac*</sub>-*lacZY*) strain (EDCM367), with selection for chloramphenicol resistance. Finally, the gene replacement by chromosomal integration and excision was carried out as described by Merlin and collaborators (18). Correct excision of the *sbcDC* operon and integration of the FLK2 cassette were verified by selection for

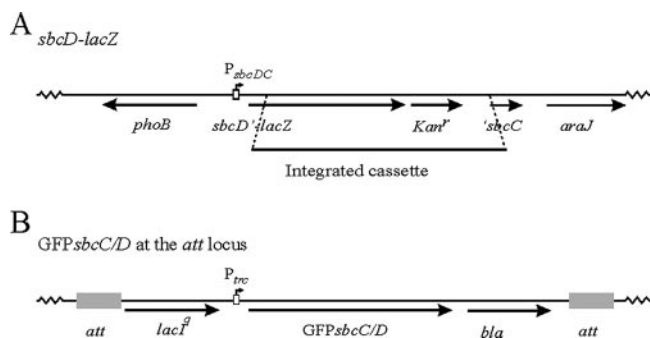


FIG. 1. Construction of mutant strains. (A) Construction of the *sbcDC-lacZ* reporter strain (DL1649). Shown is a schematic representation of the *sbcDC* region containing the  $P_{sbcDC}$ -*lacZ* reporter fusion. Part of the *sbcDC* operon was removed, and the cassette containing the *lacZ* and the kanamycin resistance genes was placed under the control of the *sbcDC* promoter region and fused to the beginning of the *sbcD* gene. *sbcD'*, 3' truncated *sbcD* gene; *Kan<sup>r</sup>*, kanamycin resistance gene; *sbcC'*, 5' truncated *sbcC* gene. (B) Construction of functional *gfp-sbcC/D* reporter strains (DL2478 and DL2479). Shown is a schematic representation of the *att* region containing the *gfp-sbcC* or *gfp-sbcD* reporter fusion. The *gfp-sbcC/D* fusion was placed under the control of the IPTG-inducible *trc* promoter region, and the protein fusion to the GFP was N terminal. *bla*, ampicillin resistance gene.

kanamycin-resistant colonies and PCR and by sequencing using primers *sbcDC2-1* and *lacZ*-*chi*-seq-R. The absence of SbcCD activity was confirmed by using a phage lambda plating test (6, 8).

The *E. coli* MG1655 *att::207NGFP-sbcC* (DL2478; Fig. 1B) and *E. coli* BW27784 *att::207NGFP-sbcC* (DL2739) strains were constructed by the insertion of an in-frame fusion of the *gfp* and *sbcC* genes under the control of an IPTG-inducible promoter into the *att* loci of *E. coli* MG1655 and *E. coli* BW27784, respectively. Insertions were carried out using the p207N*sbcC* plasmid, as described by Boyd and collaborators (3). To construct the p207N*sbcC* plasmid, the *sbcC* gene was amplified using primers NtermGFP-SbcC1 and NtermGFP-SbcC2 (3,175 nucleotides). Notably, the NtermGFP-SbcC1 primer includes a sequence introducing an ELNNN polypeptide linker between the green fluorescent protein (GFP) and the SbcC protein. The resulting PCR fragment was digested using enzymes MfeI and HindIII and cloned between the EcoRI and HindIII sites of plasmid pDSW207 (30).

The *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  *GFP-sbcC* strain (DL2533) was constructed by insertion of the *gfp* gene in frame with the *sbcC* gene using the pTOFNGFP *sbcC* plasmid. Two amplified fragments were coupled by crossover PCR and ligated into the chromosomal integration-and-excision plasmid pTOF24 (18). The first fragment of 506 nucleotides was amplified from *E. coli* MG1655 DNA using primers NtermGFP-sbcC5 and NtermGFP-sbcC6. The second fragment of 1,134 nucleotides was amplified from p207N*sbcC*, using primers NtermGFP-sbcC7 and NtermGFP-sbcC8. The 1,616-nucleotide-coupled fragment was cloned between the XhoI and SalI sites of plasmid pTOF24, resulting in the kanamycin-sensitive plasmid pTOFNGFP*sbcC*. This temperature-sensitive plasmid was introduced into the *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  strain (EDCM367) with selection for chloramphenicol resistance, and the gene replacement by chromosomal integration and excision was carried out as described by Merlin and collaborators (18). Correct integration of the *gfp* gene was verified by PCR using primers NtermGFP-sbcC5 and NtermGFP-sbcC8. The activity of the GFP-SbcC-SbcD complex was tested using the phage lambda plating test (6, 8).

The *E. coli* MG1655 *att::207NGFP-sbcD* strain (DL2479; Fig. 1B) was constructed by insertion of an in-frame fusion of the *gfp* and *sbcD* genes under the control of an IPTG-inducible promoter at the *E. coli* MG1655 *att* locus. The insertion was carried out using the p207N*sbcD* plasmid, as described by Boyd and collaborators (3). To construct the p207N*sbcD* plasmid, the *sbcD* gene was amplified using primers NtermGFP-SbcD1 and NtermGFP-SbcD2 (1,231 nucleotides). Notably, the NtermGFP-SbcD1 primer includes a sequence introducing an EFNNN polypeptide linker between the GFP and SbcD proteins. The resulting PCR fragment was cloned between the EcoRI and HindIII sites of plasmid pDSW207 (30).

The *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  *GFP-sbcD* strain (DL2535) was constructed by insertion of the *gfp* gene in frame with the *sbcD* gene using the pTOFNGFP

*sbcD* plasmid. Two amplified fragments were ligated, after PCR-mediated coupling, into the chromosomal integration-and-excision plasmid pTOF24 (18). The first fragment of 355 nucleotides was amplified from *E. coli* MG1655 DNA using primers *sbcDC2-1* and NtermGFP-*sbcD6*. The second fragment of 1,175 nucleotides was amplified from p207N*sbcD*, using primers NtermGFP-*sbcD7* and *sbcDCnd4-4*. The 1,506-nucleotide-coupled fragment was cloned between the SalI and PstI sites of plasmid pTOF24, resulting in the kanamycin-sensitive plasmid pTOFNGFP*sbcD*. This temperature-sensitive plasmid was introduced into the *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  strain (EDCM367), with selection for chloramphenicol resistance, and the gene replacement by chromosomal integration and excision was carried out as described by Merlin and collaborators (18). Correct integration of the *gfp* gene was verified by PCR using primers *sbcDC2-1* and *sbcDCnd4-4*. The activity of the SbcC-GFP-SbcD complex was tested using the phage lambda plating test (6, 8).

The *E. coli* MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *sbcDC* *rpoS359::Tn10*  $P_{sbcDC}$ -*lacZ*-*aph* (DL1718) and *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  *rpoS359::Tn10* *GFP-sbcC* (DL3117) reporter strains were constructed by transfer of the *rpoS359::Tn10* mutation from the RH90 strain into strains MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *sbcDC*  $P_{sbcDC}$ -*lacZ*-*aph* (DL1649) and MG1655  $\Delta(P_{lac-lacZY})$  *GFP-sbcC* (DL2533), respectively, using the P1 transduction technique (19).

The *E. coli* MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *sbcDC*  $\Delta$ *clpX*  $P_{sbcDC}$ -*lacZ*-*aph* (DL1796) and *E. coli* MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *clpX* (DL1913) reporter strains were constructed by deletion of the *clpX* gene from the chromosomes of the MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *sbcDC*  $P_{sbcDC}$ -*lacZ*-*aph* (DL1649) and MG1655  $\Delta(P_{lac-lacZY})$  (EDCM367) strains, using the pTOF24*clpX* plasmid. Two amplified fragments flanking the *clpX* gene were coupled by crossover PCR and ligated into the chromosomal integration-and-excision plasmid pTOF24 (18). The upstream fragment of 450 nucleotides was amplified from *E. coli* MG1655 DNA using primers *clpXflankF1* and *clpXflankR1*. The downstream fragment of 442 nucleotides was amplified from *E. coli* MG1655, using primers *clpXflankF2* and *clpXflankR2*. The 870-nucleotide-coupled fragment, after PCR-mediated coupling of these two fragments, was cloned between the SalI and PstI sites of plasmid pTOF24, resulting in the kanamycin-sensitive pTOF24*clpX* plasmid. This temperature-sensitive plasmid was introduced into *E. coli* strains MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *sbcDC*  $P_{sbcDC}$ -*lacZ*-*aph* (DL1649) and MG1655  $\Delta(P_{lac-lacZY})$  (EDCM367), with selection for chloramphenicol resistance, and the gene replacement by chromosomal integration and excision was carried out as described by Merlin and collaborators (18). Correct excision of the *clpX* gene was verified by PCR using primers *clpXflankF1* and *clpXflankR2*.

The *E. coli* MG1655  $\Delta(P_{lac-lacZY})$   $\Delta$ *clpX* *GFP-sbcC* (DL2532) strain was constructed using the pTOFNGFP*sbcC* plasmid. This temperature-sensitive plasmid was introduced into the *E. coli* strain with selection for chloramphenicol resistance, and the gene replacement by chromosomal integration and excision was carried out as described by Merlin and collaborators (18). Correct integration of the *gfp* gene was verified by PCR. The activity of the GFP-SbcC-SbcD complex was tested using the phage lambda plating test (6, 8).

To construct the *pseqA*redKm plasmid, the *seqA* gene was amplified from *E. coli* MG1655 DNA using primers *SeqA\_F* and *SeqA\_R*. The 560-nucleotide fragment was cloned between the NcoI and XhoI sites of plasmid pLAU18, resulting in plasmid p18*seqA*, which encoded a C-terminal *SeqA*-eYFP protein fusion. Subsequently, the gene encoding the DsRed protein was amplified from plasmid pRSET<sub>D</sub>DsRed DNA (5), using primers dsRED-XhoI and dsRED-HindIII. The 700-nucleotide fragment was cloned in place of the *eyfp* gene, between the XhoI and HindIII sites of plasmid p18*seqA*, resulting in *pseqA*red. Notably, the dsRED-XhoI primer includes a sequence introducing an LEL polypeptide linker between the *SeqA* and DsRed proteins. Finally, the kanamycin resistance gene was amplified from pTOF24, using primers *Km-ScaI-F* and *Km-ScaI-R*. The 837-nucleotide fragment was cloned into the *pseqA*red plasmid, using *ScaI*. The resulting plasmid *pseqA*redKm is ampicillin sensitive and kanamycin resistant.

**$\beta$ -Galactosidase activity assay.** To measure  $\beta$ -galactosidase activities, overnight cultures were diluted in fresh medium, and samples were taken at regular intervals for optical density (OD) readings at 600 nm and  $\beta$ -galactosidase activity determinations. The  $\beta$ -galactosidase assay and the calculation of  $\beta$ -galactosidase units (Miller units, nmol/OD<sub>600</sub>/min) were performed as described by Miller (19), using the following formula for calculation of the  $\beta$ -galactosidase activity:  $(OD_{420} \times 1.7)/(0.0045 \times \text{reaction time in min} \times OD_{600} \times \text{volume of cells})$ . Although some differences were observed for absolute  $\beta$ -galactosidase activities, the ratios between these activities in the various strains tested were largely constant. A ratio of about 1.5 was generally reproducible.

**Western blotting.** *E. coli* cultures were grown, and the amount of cells corresponding to an OD<sub>600</sub> of 1 was separated from the growth medium by centrifugation, resuspended in 40  $\mu$ l of fresh medium and 20  $\mu$ l of 3 $\times$  sodium dodecyl

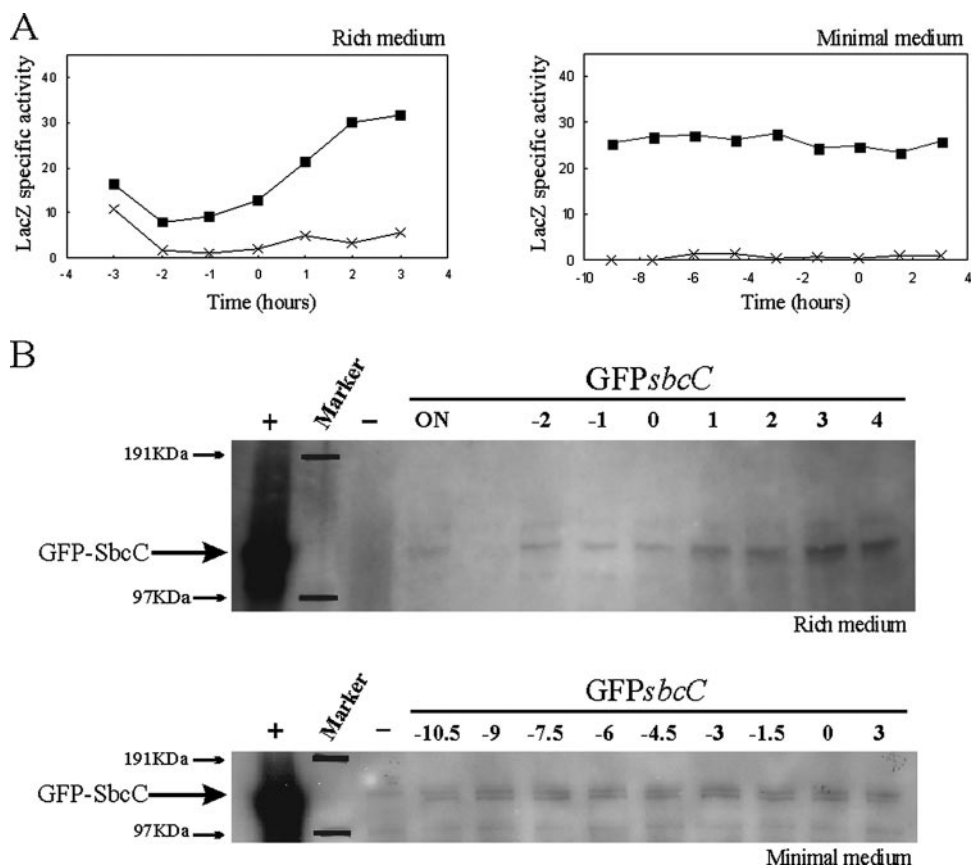


FIG. 2. Analyses of *sbcDC* transcription and SbcC protein level as a function of time and growth medium. (A) The transcriptional *sbcD-lacZ* gene fusion schematically shown in Fig. 1A was used to determine the time course of *sbcDC* expression in cells grown at 37°C in either LB (left panel) or supplemented M9 (right panel) medium. The strains used for the analyses were *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  (crosses; EDCM367) and *E. coli* MG1655  $\Delta(P_{lac-lacZY}) \Delta sbcDC P_{sbcDC-lacZ-aph}$  (closed squares; DL1649). Samples for the determination of  $\beta$ -galactosidase activities (indicated in nmol/min/OD<sub>600</sub>) were collected at defined times. Time 0 indicates the transition point between exponential and postexponential growth phases determined by a change in the slope of the growth curve. (B) GFP-SbcC fusion was used to determine by Western blotting the time course of SbcC protein levels in cells grown at 37°C in either LB or supplemented M9 medium. The position of the fusion protein is indicated. The protein marker positions were marked on the film from positions shown on the membrane. Time 0 indicates the transition point between exponential and postexponential growth phases. Times are indicated in hours. ON indicates overnight cultures. Strains used for the analyses were *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  (EDCM367; -, overnight culture as negative control), *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  GFP-*sbcC* (DL2533; GFP-*sbcC*), and *E. coli* MG1655 *atr::207NGFP-sbcC*, grown in the presence of 50  $\mu$ M of IPTG (DL2478; +, overnight culture as positive control).

sulfate sample buffer plus dithiothreitol (Biolabs), and boiled for 10 min. These preparations (10  $\mu$ l) were run on a gradient Nu-page 4 to 12% bis Tris gel (Invitrogen), and electrophoresis was performed in morpholinepropanesulfonic acid buffer, using an XCell SureLock minicell system (Invitrogen), according to manufacturer's instructions. A SeeBlue Plus2 prestained standard was used as a marker of protein size (Invitrogen original membranes were used as templates [see Fig. 2 and 3]). After proteins were separated, they were transferred to a nitrocellulose membrane (Biotrace NT), using a wet transfer Western blot apparatus (Bio-Rad) in transfer buffer (25 mM Tris, 200 mM glycine, and 20% methanol). Proteins were detected with GFP-specific rabbit antibodies (1/5,000 dilution; Invitrogen) and horseradish peroxidase anti-rabbit immunoglobulin G conjugates (1/5,000 dilution; Amersham) and visualized using an enhanced chemiluminescence solution.

**Fluorescence microscopy.** Cells were grown at 37°C in LB or supplemented M9 medium. When indicated, 50  $\mu$ M of IPTG was added to the medium (at the beginning of the day's growth for samples taken at the mid-exponential growth phase and the previous day for samples taken after an overnight culture). On the other hand, 0.001% (wt/vol) of arabinose was added to the overnight culture as well as to the day culture. Live cells were visualized on a 1% agarose-coated slide. GFP or red fluorescent protein (RFP) fluorescence was detected using a Zeiss Axiovert 200 fluorescence microscope with a GFP filter or a 4',6-diamidino-2-phenylindole (DAPI)/fluorescein isothiocyanate (FITC)/tetramethyl rhodamine isocyanate (TRITC) filter set (catalog number 82101m; Semrock). Images were

captured with a Photometrics cool-SPAP HQ charge-coupled device camera. Optical section images were collected at 150-nm intervals, deconvolved using Autovisualize+Autodeblur software (three-dimensional adaptive point spread function [blind] deconvolution), and analyzed using MetaMorph version 6 3r2 software (Molecular Devices).

## RESULTS

**Study of the transcription of the *sbcDC* operon.** In order to study the transcription of the *sbcDC* operon, a transcriptional *sbcD-lacZ* fusion was constructed in place of the native operon, inactivating both genes (DL1649; Fig. 1A). As shown in Fig. 2A, *sbcDC* transcriptional level was generally low and dependent on the growth medium (Fig. 2A). In rich medium (Fig. 2A, LB medium, left panel), *sbcDC* transcription changed as a function of growth. The transcription level was very low during exponential phase and then increased about threefold during early postexponential phase, before stabilizing at 2 h into the stationary phase. The level of *sbcDC* transcription remained low when cells were kept at exponential phase growth by reg-

ular dilution of the culture (data not shown). In minimal medium (supplemented M9 medium), the *sbcDC* transcription was constitutive (Fig. 2A, right panel). Overproduction of the SbcCD complex had no effect on *sbcDC* transcription, suggesting that the operon is not autoregulated (data not shown). Finally, the *sbcDC* transcription level was not significantly affected by a mutation in the *lexA* or the *recA* gene, indicating that the operon is not a member of the SOS regulon (data not shown).

**Study of SbcC/D protein levels.** Detection of native levels of the SbcC and SbcD proteins by using specific antibodies was unsuccessful, so GFP-*sbcC* and GFP-*sbcD* fusions were constructed at the native locus (for strains DL2533 and DL2535, respectively), and protein levels were measured by Western blotting using GFP antibodies. The normal activities of the GFP-SbcC and GFP-SbcD fusion proteins were confirmed by using a phage lambda plating test (data not shown; 6, 8). As displayed in Fig. 2B, the level of GFP-SbcC protein was generally low. Under the conditions tested, GFP-SbcC was present in cells grown in both media. GFP-SbcD was visible when cells were grown in rich medium but was barely detectable when cells were grown in minimal medium (data not shown). Surprisingly, protein levels of GFP-SbcC and GFP-SbcD were not significantly affected by the stage of cellular growth, suggesting that in rich medium, protein levels do not follow the transcription pattern (Fig. 2B and data not shown). Nevertheless, GFP-SbcC and GFP-SbcD proteins were present in the stationary phase as predicted by the transcriptional analyses.

**Effect of RpoS on *sbcDC* transcription and protein levels.** To determine whether the RpoS sigma factor is responsible for the increase of *sbcDC* transcription during postexponential phase, an *rpoS* disruption was introduced into the strain containing the *sbcD-lacZ* transcriptional fusion (DL1718). As shown in Fig. 3A, the *sbcDC* transcriptional level was 1.5-fold lower in those cells that lacked RpoS, growing in minimal medium or during the postexponential phase in rich medium (Fig. 3A). No significant differences in *sbcDC* transcription were observed during the exponential phase of cells grown in rich medium. Strikingly, in the absence of RpoS, the *sbcDC* transcriptional level in cells grown in rich medium still increased by 1.5-fold between the exponential and the postexponential phases, possibly indicating the presence of an additional regulation system. To confirm the involvement of RpoS in *sbcDC* transcription, the *clpX* gene was inactivated in the strain containing the *sbcD-lacZ* transcriptional fusion (DL1796). In the absence of ClpX, cells contained more RpoS sigma factor, and *sbcDC* transcription increased about 1.5-fold in cells grown in minimal medium or during the postexponential phase in rich medium (Fig. 3A). Notably, in rich medium, the level of *sbcDC* transcription in the *rpoS clpX* double mutant was similar to the level in the *rpoS* single mutant (data not shown). Finally, inactivation of the *hfq* gene decreased *sbcDC* transcription to a level comparable to that obtained in the absence of *rpoS* (data not shown).

Levels of SbcC and SbcD proteins were studied by Western blotting as a function of time and in the absence of RpoS or ClpX protein. As shown in Fig. 3B, GFP-SbcC protein levels, in rich or minimal medium, were similar to that in wild-type cells and in cells of strains carrying mutations in either the *rpoS* or the *clpX* gene. Furthermore, levels of GFP-SbcD were com-

parable in the presence or absence of the RpoS or the ClpX protein, when cells were grown in rich medium, while the GFP-SbcD protein was almost undetectable when cells were grown in minimal medium (data not shown).

**Localization of GFP-SbcC/D.** The GFP-*sbcC* and GFP-*sbcD* fusions used for the determination of protein levels by Western blotting were under the control of the native *sbcDC* promoter. Under these conditions, the native level of proteins was too low to permit the visualization of GFP-SbcC or GFP-SbcD by microscopy (data not shown). Strikingly, the addition of mitomycin C to the growth medium or the introduction of a palindrome into the chromosome did not permit the visualization of an eventual GFP-SbcC/D localization (data not shown). New constructs were made in order to obtain higher levels of expression of SbcCD by placing the GFP-*sbcC* or the GFP-*sbcD* fusion gene under the control of the IPTG-inducible  $P_{trc}$  promoter in the *att* locus of the *E. coli* chromosome (strains DL2478 and DL2479, respectively). Normal activities of the inducible GFP-SbcC and GFP-SbcD fusion proteins were confirmed by using the phage lambda plating test (data not shown; 6, 8). Western blotting analyses of strains containing these fusions indicated the presence of some degradation products only in those cells growing at late postexponential phase and induced by the addition of IPTG (after overnight culture; data not shown).

Localization of the GFP-SbcC and GFP-SbcD proteins using these new constructs was determined under different conditions by using microscopy (Fig. 4). In the absence of IPTG, cell fluorescence was very low, close to the background level, and no localization was perceptible. After the addition of IPTG, foci were visible in exponential phase cells expressing the GFP-SbcC fusion protein. When cells were grown in minimal medium, most of these cells displayed either a mid-cell focus or two foci, one located at one-quarter and the second at three-quarters of the cell length. More foci with different intensity levels were visible in cells grown in rich medium. During stationary phase, overproduction of GFP-SbcC in cells grown in rich medium led to the presumed artifactual accumulation of fusion proteins at cellular poles, whereas when cells were grown in minimal medium, GFP-SbcC seemed to be evenly distributed throughout the cytoplasm. Interestingly, the GFP-SbcC cellular localization was independent of the presence and the level of the SbcD protein (data not shown). Conversely, when IPTG was added to cells expressing the functional GFP-SbcD fusion, fluorescence was evenly distributed throughout their cytoplasm, independently of the growth medium or the growth phase (Fig. 4). Finally, the cytoplasmic distribution of GFP-SbcD subsisted in the absence of the SbcC protein (data not shown).

**Colocalization of the GFP-SbcC and RFP-SeqA proteins.** The localization of GFP-SbcC in exponential phase cells grown in minimal medium was similar to the documented localization of the cell replication factory (20). To determine whether the SbcC protein might be associated with the replication factory, GFP-SbcC fusion protein localization was studied in association with a protein that binds DNA at replication forks, SeqA. The *pseqAredKm* plasmid carrying an arabinose-inducible RFP-SeqA fusion protein was introduced into a strain containing the IPTG-inducible GFP-*sbcC* fusion gene (strain DL2739). Notably, homogeneous expression from the arabi-

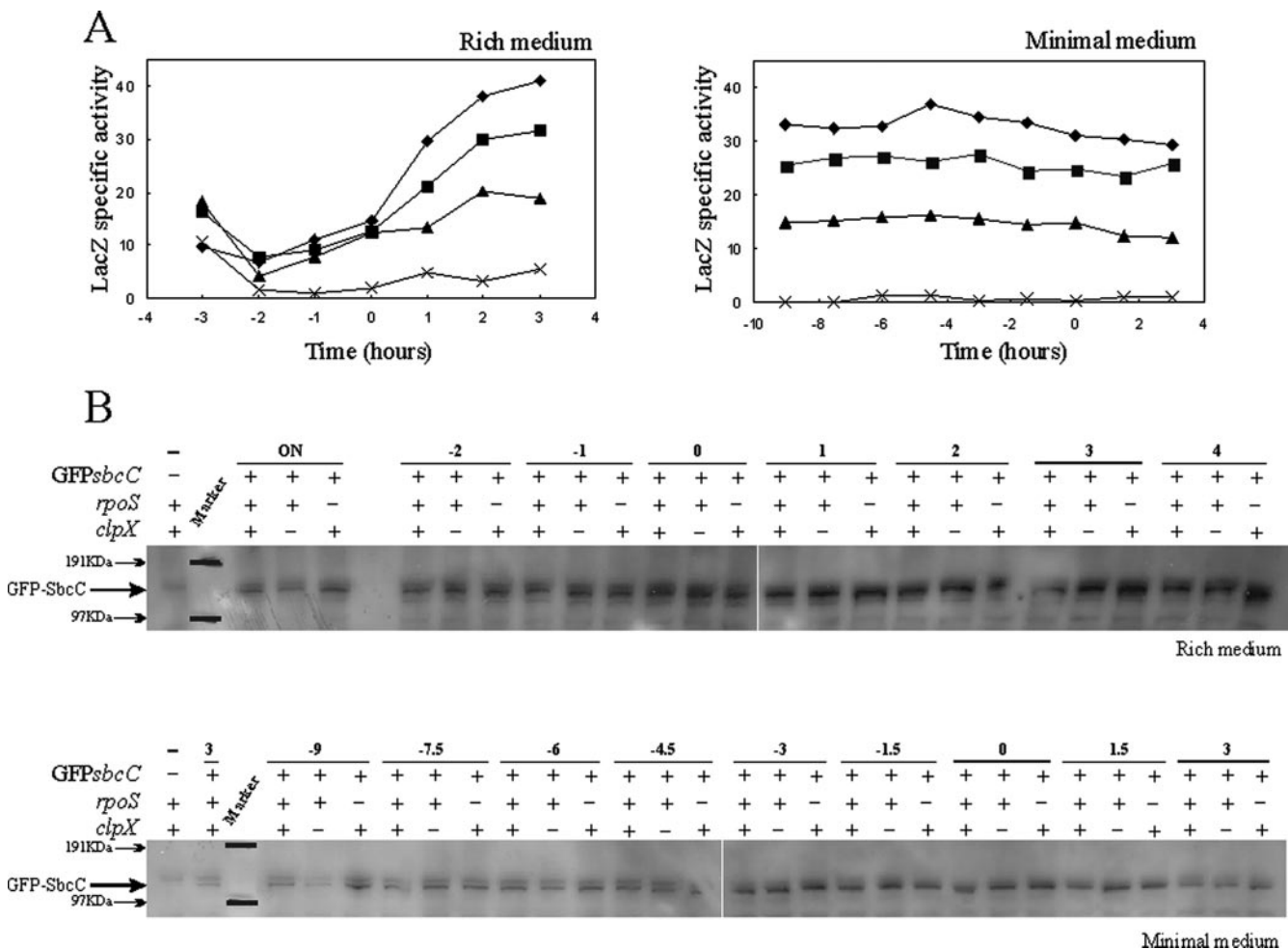


FIG. 3. Effect of the *rpoS* or *clpX* mutation on *sbcDC* transcription and SbcC protein level. (A) The transcriptional *sbcDC-lacZ* gene fusion, schematically shown in Fig. 1A, was used to determine the time course of *sbcDC* expression in the presence and absence of the *rpoS* or *clpX* gene in cells grown at 37°C in either LB (left panel) or supplemented M9 (right panel) medium. Strains used for the analyses were *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  (*rpoS* mutant; EDCM367), *E. coli* MG1655  $\Delta(P_{lac-lacZY}) \Delta sbcDC P_{sbcDC-lacZ-aph}$  (*clpX* mutant; DL1649), *E. coli* MG1655  $\Delta(P_{lac-lacZY}) \Delta sbcDC rpoS359::Tn10 P_{sbcDC-lacZ-aph}$  (*rpoS* mutant; DL1718), and *E. coli* MG1655  $\Delta(P_{lac-lacZY}) \Delta sbcDC \Delta clpX P_{sbcDC-lacZ-aph}$  (*clpX* mutant; DL1796). Samples for the determination of  $\beta$ -galactosidase activities (indicated in nmol/min/OD<sub>600</sub>) were collected at defined times. Time 0 indicates the transition point between the exponential and postexponential growth phases determined by a change in the slope of the growth curve. (B) GFP-SbcC fusion was used to determine by Western blotting the time course of SbcC protein level in the presence and absence of RpoS or ClpX protein in cells grown at 37°C in either LB (top panel) or supplemented M9 (lower panel) medium. The position of the fusion protein is indicated. The protein marker positions were marked on the film from positions shown on the membrane. Time 0 indicates the transition point between the exponential and postexponential growth phases. Times are indicated in hours. ON indicates overnight cultures. The strains used for the analyses were *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  (EDCM367; -, overnight culture as negative control), *E. coli* MG1655  $\Delta(P_{lac-lacZY})$  GFP-*sbcC* (DL2533), *E. coli* MG1655  $\Delta(P_{lac-lacZY}) \Delta clpX$  GFP-*sbcC* (DL2532), and *E. coli* MG1655  $\Delta(P_{lac-lacZY}) rpoS359::Tn10$  GFP-*sbcC* (DL3117).

nose-inducible system was ensured by using a specific background strain (BW27784 [11]). Colocalization of these two fluorescent proteins was studied in 777 exponential phase cells grown in minimal medium, distributed in three independent experiments. Notably, 56% of these cells displayed one GFP-SbcC focus, and 34% displayed several foci; so, only 10% of the cells showed a dispersed cytoplasmic localization of GFP-SbcC. On the other hand, 48% of cells displayed one RFP-SeqA focus, and 20% displayed several foci; so, 32% of cells showed a dispersed RFP-SeqA cytoplasmic localization. The fluorescence of the RFP-SeqA fusion protein was low, and foci were difficult to visualize, probably explaining the fact that cells

seem to have less RFP-SeqA foci than GFP-SbcC foci. Interestingly, in the 510 cells displaying at least one focus of each GFP-SbcC and RFP-SeqA fusion protein, colocalization was observed in 94% of cells, with 79% of foci showing colocalization. Figure 5 illustrates some GFP-SbcC/RFP-SeqA colocalizations. This result suggests that GFP-SbcC and RFP-SeqA are closely associated.

**DISCUSSION**

This work presents, for the first time in *E. coli*, a study of the SbcCD regulation and localization. The transcription of the

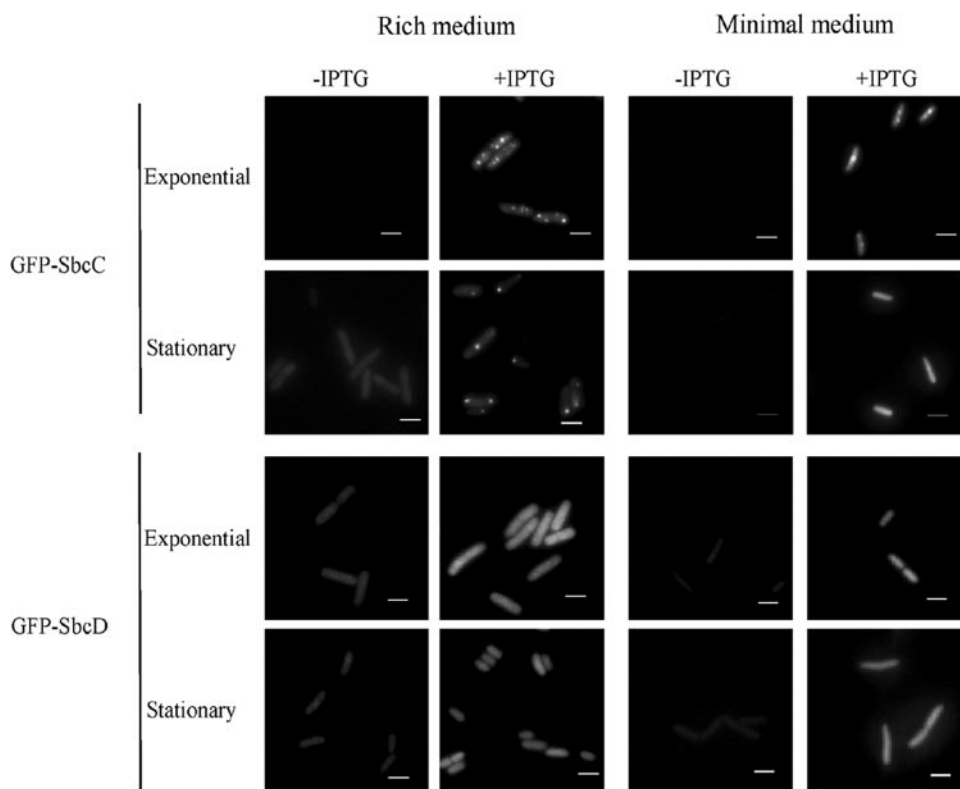


FIG. 4. Localization of GFP-SbcC/D fusion proteins as a function of time and growth medium. *E. coli* MG1655 *att::207NGFP-sbcC* (DL2478; GFP-SbcC) and *E. coli* MG1655 *att::207NGFP-sbcD* (DL2479; GFP-SbcD) were grown at 37°C in either LB (left panels) or supplemented M9 (right panels) medium in the presence or absence of 50  $\mu$ M of IPTG. Cells were grown either until mid-exponential growth phase or until stationary phase (overnight) and then visualized on a 1% agarose-coated slide by fluorescence microscopy using a GFP filter. The calibration bar indicates 2  $\mu$ m.

*sbcDC* operon is induced in an RpoS-dependent manner when cells are under starvation conditions or at postexponential phase in rich medium. However, for an unknown reason, the SbcC and SbcD protein levels do not seem to follow the transcription level. The natural levels of SbcC and SbcD are low, and GFP fusion proteins needed to be overexpressed to be visualized. Surprisingly, the two proteins do not colocalize as SbcC is associated with the replication factory, and SbcD is dispersed throughout the cytoplasm.

The action of RpoS on *sbcDC* transcription is probably indirect as the effect is significant but low, and the *sbcDC* promoter does not contain a  $\sigma^S$ -specific consensus sequence (29). On the other hand, the *sbcDC* promoter presents a perfect  $-10$  region for the housekeeping  $\sigma^{70}$  factor (21). To work efficiently, the  $\sigma^{70}$  factor also needs a  $-35$  region, which is absent from the *sbcDC* promoter. However, the *sbcDC* promoter seems to display a proximal site of an UP element consensus sequence that can bind the  $\alpha$  subunit of RNA polymerase and help to stimulate transcription of the operon in the absence of a  $-35$  box (26). Altogether, these observations could explain the low level of transcription of the *sbcDC* operon. Notably, the effect of RpoS on *sbcDC* transcription is probably too small to have been detected in previous studies using general methods such as DNA array analysis or random *lacZ* fusion mutagenesis (23, 28, 29). The fact that RpoS does not act directly on the *sbcDC* promoter suggests the presence

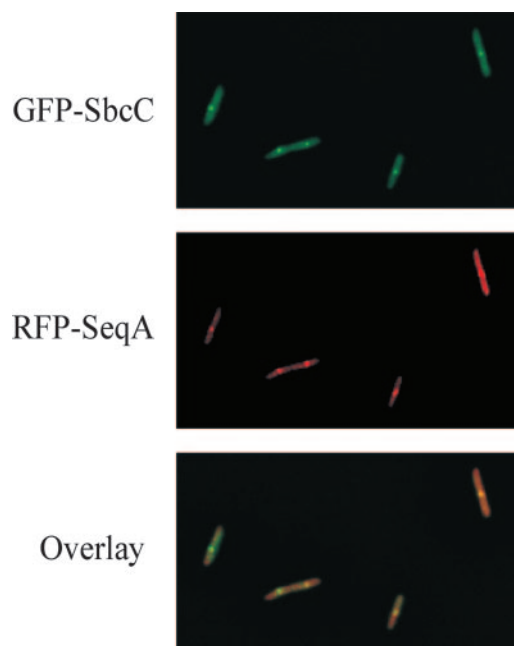


FIG. 5. Colocalization of GFP-SbcC and RFP-SeqA fusion proteins. *E. coli* BW27784 *att::207NGFP-sbcC* carrying the *pseqAredKm* plasmid (DL2739 plus *pseqAredKm*) was grown at 37°C in supplemented M9 medium in the presence of 50  $\mu$ M of IPTG and 0.001% of arabinose until mid-exponential growth phase. Cells were visualized on a 1% agarose-coated slide by fluorescence microscopy using a DAPI/FITC/TRITC filter set.



of another regulatory system. Similarly, RpoS does not seem to totally control *sbcDC* transcription, as the operon expression still increases when *rpoS* mutant cells enter stationary phase. Random mutagenesis was unsuccessfully employed to investigate the identity of this potential regulator (data not shown). The unfound regulatory system might be essential or not be a protein, or the screen employed was not sensitive enough.

Surprisingly, GFP-SbcC and GFP-SbcD protein levels did not reflect the transcription pattern of the operon. Western blotting of GFP fusion proteins might not have been sensitive enough to detect the small differences seen in transcriptional levels, or the fusions might affect the stability of the proteins. Another explanation could be the presence of an additional SbcCD regulation at the mRNA or protein level. There are no published studies on the regulation of the *sbcDC* mRNA in *E. coli*. General studies of protein-protein interactions in *E. coli* found only one protease, ClpA, that has a potential interaction with SbcC (1, 4). However, this interaction was not validated, and ClpA did not appear to interact with the SbcD protein.

The presence of SbcCD at stationary phase is unexpected as the function of the proteins has been associated with DNA repair in the context of replication. This work suggests that SbcCD must have some as-yet unknown function in the stationary phase or in the exit from stationary phase.

The SbcC protein seems to localize at replication forks, whereas SbcD seems to be evenly distributed throughout the cytoplasm. This suggests either that SbcC and SbcD can function independently of each other or that the regulation of activity is achieved by ensuring that the majority of the two proteins are not associated at any one time. The function of SbcC could be to recognize an SbcD substrate and modulate the nuclease activity of SbcD. SbcC might constantly check the replication fork for misfolded DNA and associate with SbcD only when DNA repair is necessary. SbcD action might require too small an amount of protein or might be too quick to be visualized by microscopy.

Interestingly, overproduced SbcC and SbcD proteins in *E. coli* and *B. subtilis* localize in similar ways, but the localization and regulation of the natural level of protein are different between the two organisms (16, 17). A native level of SbcC localized as discrete foci in 2% of *B. subtilis* cells but could not be visualized in *E. coli* (16). Additionally, the SbcC and SbcD protein levels in *B. subtilis* were induced when mitomycin C was added to the cells, while this drug had no effect on *E. coli* *sbcDC* transcription (16; data not shown). After the addition of mitomycin C, up to 40% of *B. subtilis* cells displayed SbcC foci, whereas no SbcC focus was visible in *E. coli* cells (data not shown). The presence of foci at a natural level of SbcC in *B. subtilis* but not in *E. coli* could be explained by a higher production of SbcC protein in *B. subtilis* than in *E. coli*. Notably, in the two organisms, overexpressed SbcC protein localizes as does native SbcC in *B. subtilis*. Therefore, SbcC might always localize at the replication fork, but the natural level of the protein is too low to be seen by microscopy in *E. coli*. The addition of mitomycin C in *B. subtilis* culture increases the SbcC protein level, permitting the visualization of more foci. The differences in SbcCD regulation between the two organisms could be explained by a different function of the complex. Interestingly, *B. subtilis* but not *E. coli* contains proteins that are involved in the nonhomologous end-joining DNA repair pathway (31), and

SbcCD homologues in eukaryotes may play a role in this pathway (22).

In conclusion, this work increases the understanding of SbcCD in *E. coli* by investigating its regulation and localization. However, the exact role of this complex is still to be revealed and seems to be variable in different organisms.

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