SecA defects are accompanied by dysregulation of MukB, DNA gyrase, chromosome partitioning and DNA superhelicity in Escherichia coli.
**SecA defects are accompanied by dysregulation of MukB, DNA gyrase, chromosome partitioning and DNA superhelicity in *Escherichia coli***

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Abstract

Spatial regulation of nucleoids and chromosome partitioning proteins is important for proper chromosome partitioning in *Escherichia coli*. However, the underlying molecular mechanisms are unknown. In the present work, we show that mutation or chemical perturbation of SecA (*Secretory A*: an ATPase component of the membrane protein translocation machinery), SecY (a component of the membrane protein translocation channel), and AcpP (*Acyl carrier protein P*), which binds to SecA and MukB (functional homologue of *Structural Maintenance of Chromosomes* protein, SMC), resulted in a defect in chromosome partitioning. We further show that SecA is essential for proper positioning of the oriC DNA region, decatenation, and maintenance of superhelicity of DNA. Genetic interaction studies revealed that the topological abnormality observed in the secA mutant was due to combined inhibitory effects of defects in MukB, DNA gyrase, and Topo IV, suggesting a role for the membrane protein translocation machinery in chromosome partitioning and/or structural maintenance of chromosomes.
INTRODUCTION

The mechanisms of chromosome partitioning in bacterial cells are not known. Currently, two general issues are important in addressing the problem (Fisher et al., 2013): (I) how the cell places sister chromosomes in distinct spaces, also named ‘positioning’; (II) the nature of the energy that is required for the process. In this paper, we mainly investigated (I), the nature of ‘positioning’, and describe the underlying mechanisms involved in the general topology of chromosomes.

In Escherichia coli, the replication origin of chromosome (oriC) localizes at mid-cell before the onset of replication, and then the replicated sister oriC copies move to the quarter cellular positions after duplication. Similarly, MukB ("mukaku" means anucleate in Japanese), which is a functional homologue of SMC (Structural Maintenance of Chromosomes) that mediates chromosome organization, localizes equidistantly along the long cell axis as foci in living cells (Hiraga, 2000; Ohsumi et al., 2001; Adachi et al., 2008; for a review, see Nolivos & Sherratt, 2013). In ∆mukB (mukB null-deletion) mutant cells, localization of
the oriC region, nucleoids, and replication forks is affected, and these mutant cells exhibit a defect in separation of sister chromosomes at the restrictive temperature (at or above 30°C); this is referred to as the Par− (Partition minus) phenotype (Niki et al., 1991). These observations indicate that positioning of these factors through spatial regulation may play important roles in chromosome partitioning (defined as separation of sister chromosomes), but the underlying molecular mechanism of this process is not yet clear.

DNA gyrase and DNA topoisomerase IV (Topo IV), which are involved in topological regulation of DNA, are also critical for chromosome partitioning. Similar to MukB, inactivation of DNA gyrase or Topo IV also results in the Par− phenotype (Wang, 2002). Interestingly, MukB and DNA gyrase functionally interact with each other (Adachi & Hiraga, 2003). MukB and Topo IV also functionally and physically interact (Hayama & Marians, 2010; Li et al., 2010; Hayama et al., 2013). Thus, the topology of the nucleoid or chromosomal region is thought to be important for proper chromosome partitioning in bacteria.
In *E. coli*, Sun & Margolin (2004) briefly described the Par\(^{-}\) phenotype in SecA (Sec*tery A*) conditional mutant cells, which they used to address the function of FtsZ (tubulin homologue). We are interested in the underlying molecular mechanism that causes the chromosome partitioning defect in the absence of SecA. SecA mediates translocation of membrane proteins through the Sec pore complex of the inner cell membrane, thus maintaining homeostatic conditions in cells via the membrane transport machinery (Roboson & Collinson, 2006). SecY is a component of the membrane protein translocation channel together with SecE and SecG (Oliver *et al.*, 1990). SecY and SecB interact with SecA and stimulate the ATPase activity of SecA (Miller *et al.*, 2002; Natale *et al.*, 2004). Therefore, we also investigated the molecular link between SecA and proteins known to be involved in chromosome partitioning such as MukB, DNA gyrase, and Topo IV.
In the present work, we demonstrate the roles of SecA - SecY (a component of the membrane protein translocation channel) and also AcpP, which is an interacting partner of SecA, in proper chromosome partitioning.

AcpP is involved in fatty acid biosynthesis. AcpP is the sole essential acyl carrier protein in *E. coli* and is acylated during fatty acid biosynthesis as a carrier and donor (De Lay & Cronan, 2006). However, AcpP is also a hub protein in *E. coli* and interacts with MukB as detected with both co-purification (Niki *et al.*, 1992) and high-throughput analysis (Butland *et al.*, 2005). AcpP also interacts with proteins not related to lipid synthesis, such as DNA polymerase III, RNA polymerase, and ribosomes, suggesting a variety of functions and the possibility of a role in chromosome partitioning when working together with MukB. Finally, we found functional epistasis of SecA over MukB, DNA gyrase, and Topo IV, revealing a novel role for the membrane protein translocation machinery in chromosome partitioning and/or chromosome organization.
METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1 and Table S1, respectively. Derivatives that have the azide-resistant secA204 mutation were obtained by P1 transduction (using P1 vir) from W208 and were selected and purified with 3-4 mM sodium azide. Derivatives that have the ΔmukB mutation were obtained with co-transduction with the kanamycin resistance gene from AZ5372 and were selected and purified with 30 µg/ml kanamycin.

Media. Medium L (Hiraga et al., 1989) consists of 1% Bactotryptone (Difco, Sparks, MD), 0.5% yeast extract (Difco), and 0.5% NaCl (pH 7.4). Medium RB was used to grow the temperature-sensitive acpP mutant according to the original paper (Davis et al., 1982). Medium RB consists of 1% Bactotryptone (Difco), 0.1% yeast extract (Difco), and 0.5% NaCl. Medium C consists of synthetic medium M9 (Miller, 1992) supplemented with 0.5% glycerol, 50 µg/ml L-
threonine, L-leucine, L(−)-proline, L-arginine, L-histidine, L-tryptophan, and 5 µg/ml vitamin B1. Ampicillin (50 µg/ml), kanamycin (30 µg/ml), and gentamycin (10 µg/ml) were added to the cultures of strains when necessary. For strain IL05 (pLAU53), L-(+)-arabinose (0.01%), anhydrotetracycline (12 nM), and isopropyl β-D-1-thiogalactopyranoside (0.5 mM) were added to the culture (Lau et al., 2003).

Fluorescence Microscopy. Microscopy was performed as described in Adachi et al. (2008). When necessary, cells were stained with DAPI (4',6-diamidino-2-phenylindole) and observed according to the fluorescence-phase combined method described by Hiraga et al. (1989).

Protein translocation assay in vivo. The protein translocation assay in vivo was performed according to Oliver et al. (1990). Bacterial cultures were grown at 30°C in M9 medium containing 0.5% maltose, 50 µg/ml L-threonine, L-leucine, L(−)-proline, L-arginine, L-histidine, L-tryptophan, and 5 µg/ml vitamin
B1. Ampicillin (50 µg/ml), kanamycin (30 µg/ml), and gentamycin (10 µg/ml) were added when necessary. In the mid-exponential phase, 1 mM sodium azide was added when necessary and incubated for another 10 min. Then, 20 µCi of a mixture of 73% L-[35S]methionine and 22% L-[35S]cysteine (>1000 Ci/mmol; PerkinElmer, Inc., Waltham, MA) were added to 1 ml of the culture and incubated for 5 min (30°C), followed by addition of an equal volume of ice-cold 10% trichloroacetic acid. Samples were incubated on ice for 15 min, centrifuged for 45 min at 4°C, 15,000 × g, and washed with acetone. Then we performed immunoprecipitation analysis as described (Kruse et al., 2006). Samples were suspended in 80 µl lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Nonidet, and a cocktail of EDTA-free protease inhibitors; Nacalai Tesque, Inc., Kyoto, Japan), placed on ice for 20 min, sonicated, and cell debris was removed by centrifugation at 15,000 × g for 30 min at 4°C. Cleared cell lysates were incubated for 30 min at 4°C with pretreatment resin, and then the supernatants were incubated for 4 hours at 4°C with mouse anti-maltose binding protein (MBP) monoclonal antibody (Zymed® Laboratories, Inc., San Francisco, CA).
and protein G sepharose beads (GE Healthcare U.K., Ltd., Little Chalfont, UK). Precipitated immune complexes were washed five times with lysis buffer and eluted with SDS-lysis buffer. The samples were electrophoresed on 10% polyacrylamide/0.13% bisacrylamide gels, fixed in 10% glacial acetic acid for 30 min, rinsed in water for 30 min, soaked in 1 M sodium salicylate (pH 6.0) for 1 hour, dried, and exposed to film at room temperature.

Analysis of the superhelicity of plasmid DNA molecules with two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to the previous work (Weitao et al., 2000). Cultures (400 ml) of bacterial strains carrying the plasmid pUC19 were exponentially grown in medium C and harvested at OD$_{600} = 0.6$. Plasmid DNA was extracted using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, Corp., Carlsbad, CA) and stored in 0.2 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at −20°C. The first-dimension run for the horizontal 2D electrophoresis was performed in 0.8% agarose in TAE buffer at 2.5 V/cm for 3 hours. Then, the gel was turned 90°, and
soaked for 3 hours in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) containing 25 µg/ml (~80 µM) chloroquine. Then, the second-dimension run was performed at 1 V/cm for 18 hours, resulting in higher mobility for DNA with less negative supercoiling (Hinton et al., 1992). Plasmid DNA was detected by staining with 1 µg/ml ethidium bromide after the electrophoresis.

**Immunofluorescence microscopy.** Indirect immunofluorescence microscopy using SeqA antiserum and goat anti-rabbit IgG antiserum was performed as described previously (Hiraga et al., 1998). We used goat anti-rabbit IgG antiserum conjugated to Cy3 (GE Healthcare, U.K. Ltd.) as the secondary antibody.

**Run-off replication method to analyze the DNA content per cell using flow cytometry.** This procedure was performed as described (Adachi et al., 2008).
RESULTS

Sodium azide and mutations in secA, secY, and acpP affect the morphology of the nucleoid

Because the secA mutant exhibits the Par− phenotype (Sun & Margolin, 2004), we attempted to recapitulate the Par− phenotype. When temperature-sensitive (Ts) secA51 mutant cells (MM52) were incubated at the non-permissive temperature of 42°C in medium L for 4 hours, nucleoids in 100% (36/36) of cells were elongated and enlarged, showing the typical Par− phenotype, compared to the isogenic wild-type strain (MC4100) that did not exhibit any abnormalities (0/149, Fig. 1a). This is consistent with a previous report that briefly described the Par− phenotype in a secA mutant (Sun & Margolin, 2004). We further asked whether inactivation of other components of the membrane protein translocation machinery such as SecY (a membrane protein component of the translocation channel) also resulted in the Par− phenotype. When cold-sensitive (Cs) secY39 mutant cells (AD208) were incubated in medium L at the restrictive temperature
of 22°C for 20 hours, 83% (52/63) of cells exhibited the typical Par− phenotype (Fig. 1b). The morphologies of the cells and the nucleoid were normal in both mutants at permissive temperatures 30°C and 37°C (1/186 and 4/64 abnormalities), respectively. At all temperatures tested, isogenic wild-type strains (MC4100 and TYO) showed normal morphology (1/286 and 4/58 abnormalities).

In addition, we tested a temperature-sensitive acpP mutant (NRD29) and observed that these cells also exhibited the Par− phenotype at a frequency of ~70% (86/125) after incubation at the non-permissive temperature of 42°C for 3 hours (Fig. 1c), but not at the permissive temperature of 30°C (2/109) or in the isogenic wild-type strain (0/206) (NRD52). The result was similar to that of the ∆mukB mutant at 42°C (see INTRODUCTION).

We next sought to independently recapitulate the Par− phenotype by chemically blocking the ATPase activity of SecA using 1 mM sodium azide (Oliver et al., 1990). Among many essential ATPases in vivo, the SecA ATPase is the sole target
of 1 mM sodium azide because an azide-resistant secA204 mutant (MQ597) grew exponentially in the presence of 1 mM sodium azide and grew as well as the mutant in the absence of the chemical (Fig. 1d). On the other hand, the isogenic strain with the wild-type secA allele (IL05/pLAU53) grew exponentially for 2 hours after the addition of sodium azide, and then the growth was gradually inhibited (Fig. 1d). Furthermore, the nucleoid morphology observed in the mutant grown in 1 mM sodium azide was entirely normal (99/99 control cells and 71/71 azide-treated cells; Fig. 1e). We believe that observed effects of sodium azide are due only to inactivation of SecA ATPase activity, and not to other effects at this low concentration. When IL05 (pLAU53) cells were incubated with 1 mM sodium azide for 4 hours at 30°C in medium C (minimal medium with a few supplementary amino acids), approximately 30% (from 4/602 at 0 hours to 178/626 at 4 hours) of cells showed the typical Par− phenotype (Fig. 1e). The proportion of elongated cells increased with further incubation. Similar results were obtained in medium L (rich medium, from 0/131 at 0 hours to 70/107 at 4
hours), in which cells were larger and the Par\textsuperscript{−} phenotype became easier to
observe (Fig. 1f).

The cell elongation phenotype with a large nucleoid(s) is not due to the SOS
(Morse code) response, which is caused by inhibition of chromosomal DNA
replication, because induction of $\lambda$ phage (a response of SOS) was not observed
in the $\lambda$-phage lysogenized wild-type secA strain (MQ504) in the presence of 1
mM sodium azide or in the $\lambda$-phage lysogenized secA$^{51}$ mutant (MQ508) at the
non-permissive temperature (Fig. S1). As a positive control, mitomycin C (1
$\mu$g/ml), which causes DNA damage, was used to induce $\lambda$-phage induction of the
strain tested (Fig. S1). Addition of 1 mM sodium azide did not inhibit ongoing
replication or initiation of chromosomal DNA replication (Supplementary Text,
Fig. S2).

Involvement of SecA in intracellular positioning of oriC
We further investigated the cause of the chromosome partitioning defect in the secA mutant. Because spatial regulation of nucleoids and chromosome partitioning proteins is considered to be important for proper chromosome partitioning, we examined the effect of SecA on the positioning of oriC. The oriC region and other chromosomal loci are known to localize as foci, and their intracellular positioning is strictly regulated (Hiraga, 2000), rendering oriC a suitable system for quantitatively detecting positioning defects during chromosome positioning.

We used the IL05 (pLAU53) strain, which contains 240 repeats of the tetO insertion near oriC that can be visualized as fluorescent foci by TetR-EYFP (Lau et al., 2003). Cells of this strain grew exponentially with a doubling time of 150 min in medium C at 30°C. Samples were taken at various intervals and observed with a fluorescence microscope for oriC foci. According to Bates and Kleckner (2005), the oriC locus localizes at a new pole-biased region at the beginning of the normal cell cycle. The locus then moves to mid-cell, begins replicating, and
after a brief cohesion, the focus splits into two. One remains at mid-cell, and the other moves towards the 1/4 position in length. Thereafter, the mid-cell focus moves towards the 3/4 position, and each locus remains at its corresponding position until cell division. We normalized the cell length and divided it into five zones as shown in Fig. 2b. Then, the intracellular localization of oriC foci was classified into 12 types as shown in Fig. 2a. Because oriC never localizes very close to cell poles in the normal Bates and Kleckner scheme, these 12 types were grouped into two groups, “irregular-type (polar-type)” (Types 1C, 2E, 2F, 2G, 2H, & 2I) and “regular-type (nonpolar-type)” (the remaining types), depending on whether at least one oriC focus was abnormally localized at a cell pole. Before inhibition of SecA with sodium azide, we found fewer than 2% of irregular-type cells in the sample. However, irregular-type cells increased to approximately 20% 1 hour after the addition of 1 mM sodium azide (Fig. 2a, c).

As shown in Figure 2d and e, in the azide-resistant secA204 mutant (MQ597), irregular-type cells were observed at high frequency (approximately 15%), even
in the absence of sodium azide. In the membrane translocation assay with MBP, the precursor of MBP (pre-MBP), which was larger than the membrane-translocated mature MBP lacking the signal peptide, was not observed in the secA204 mutant cells grown in either the absence or presence of 1 mM sodium azide (Fig. 2f, lanes 3 and 4). This indicates that the mutant has normal translocation activity for membrane proteins in both growth conditions. This is consistent with previous results reported by Oliver et al. (1990) who showed that the secA204 mutation does not affect SecA ATPase activity or the translocation activity for MBP in vivo. Thus, although the altered SecA protein in the mutant has normal translocation activity for membrane proteins, the protein is defective in its ability to affect oriC positioning.

**SecA affects the topology of plasmid DNA**

Topological regulation of nucleoids or chromosomal regions is considered to be important for proper chromosome partitioning in bacteria because proteins involved in topological regulation of DNA such as MukB, DNA gyrase, and Topo
IV are critical for partitioning of nucleoids. We asked if inactivation of SecA results in abnormal topology of DNA, which is due to a failure in MukB, DNA gyrase, and Topo IV functions. For this purpose, we used plasmid assays to monitor the topology of DNA in a series of mutants.

Strains harboring high-copy pUC19 plasmid were incubated in medium C, and the topology of the plasmids was assessed with two-dimensional gel electrophoresis. We first analyzed plasmid topology in the temperature-sensitive secA51 (Ts) mutant (MM52), which exhibits the Par− phenotype. Notably, when the secA51 mutant cells were incubated at the non-permissive temperature of 42°C for 2 hours, covalently closed circular monomer plasmid molecules moved more rapidly (Fig. 3b, b’) than those of the wild-type secA strain (MC4100) (Fig. 3a, a’) in the second-dimension electrophoresis. This shows that the inactivation of SecA function reduced the superhelicity of plasmid DNA, suggesting inactivation of DNA gyrase, which increases superhelicity of plasmid DNA molecules (Weitao et al., 2000). Furthermore, additional spots were observed that
slowly moved in the first-dimension electrophoresis (Fig. 3b, b’). These spots presumably corresponded to plasmid concatemers.

As a control experiment, we examined the effect of inhibiting DNA gyrase on plasmid topology. Novobiocin is primarily an inhibitor of DNA gyrase at a concentration of 100 µg/ml \textit{in vivo} (Adachi & Hiraga, 2003). When cells of the wild-type strain (MC4100) were incubated in the presence of novobiocin (100 µg/ml) for 6 hours, covalently closed circular monomers of plasmid DNA moved more rapidly in the second-dimension electrophoresis (Fig. 3c, c’) than those in cells grown without novobiocin treatment (Fig. 3a, a’), indicating the expected reduction in superhelicity. Additional spots presumably corresponding to concatemers moved slowly in the first-dimension electrophoresis. We next examined plasmid molecules in the $\Delta$\textit{mukB} mutant (MQ761) at the non-permissive temperature of 42°C for 2 hours. As shown in Fig. 3d and d’, we found that covalently closed circular monomers moved the same as those in the wild-type strain (Fig. 3a, a’), suggesting that the \textit{mukB} mutation did not markedly
affect the superhelicity. However, many additional spots were observed that corresponded to concatemers of plasmid DNA monomers, which moved slowly in the first-dimension electrophoresis.

We speculate that the defects in the SecA function in plasmid topology are synthetic effects due to defects in DNA gyrase and MukB. \( \Delta mukB \) mutant cells are hypersensitive to novobiocin (Weitao et al., 1999; Onogi et al., 2000). Novobiocin at 100 \( \mu \)g/ml is a lethal dose in \( \Delta mukB \) mutant cells, but not in wild-type cells (Adachi & Hiraga, 2003). This concentration of novobiocin inhibits separation (decatenation) of sister chromosomes in the \( \Delta mukB \) mutant. Therefore, we used this concentration to further study the effect of novobiocin in lethality.

When \( \Delta mukB \) mutant cells were treated with 100 \( \mu \)g/ml novobiocin for 4 hours followed by a shift up to the non-permissive temperature of 42°C for 2 hours, covalently closed circular monomers moved rapidly in the second-dimension electrophoresis (Fig. 3e, e’), similar to the \( secA51 \) mutant at the non-permissive temperature (Fig. 3b, b’). A large number and high amount of additional spots
corresponding to concatemers were also observed in this condition, the same as
the secA51 mutant at the non-permissive temperature.

To confirm that SecA affects the chromosome partitioning pathway upstream of
DNA gyrase and MukB, we tested the secA51 ΔmukB double mutant (MQ760)
incubated at the non-permissive temperature of 42°C both in the presence (Fig. 3f,
f’) and the absence (Fig. 3g, g’) of novobiocin. The patterns observed were the
same as those observed in the secA51 single mutant (Fig. 3b, b’). This result
suggests epistasis of secA over mukB and gyrB (DNA gyrase B subunit gene,
target of novobiocin).

Topo IV is known to relax the superhelicity of plasmid DNA molecules and to
decatenate DNA concatemers. As another control, we examined the effect of
inactivation of Topo IV on plasmid topology. We used a temperature-sensitive
parC1215 mutant (EJ812) with a mutation in the gene encoding the Topo IV
subunit ParC. When the parC1215 mutant cells were incubated at the non-
permissive temperature of 42°C for 2 hours, covalently closed circular monomer molecules from the mutant cells were detected as three spots that moved more slowly (Fig. 3h, h’) than covalently closed circular monomers from wild-type cells (Fig. 3a, a’) in the second-dimension electrophoresis as expected, suggesting an increase in negative superhelicity at three different levels. The negative superhelicity of the plasmids in the other spots was also increased, suggesting inactivation of the relaxation activity of Topo IV. In this condition, the amount of plasmid concatemer is less than 10% (Zechiedrich & Cozzarelli, 1995), indicating that very few concatemers are visualized in the gel.

The temperature difference between 42°C and 30°C did not significantly affect the pattern of the plasmid topology in the wild-type strain (Fig. 3i, i’, a, a’), indicating that the temperature variations used in this study did not affect DNA topology.
DISCUSSION

In the present work, we found evidence that links SecA to chromosome partitioning proteins: (1) the deficiency in SecA, as well as SecY and AcpP, causes a chromosome partitioning defect; (2) SecA is essential for proper localization of the oriC locus; (3) deficiency in secA causes alteration of DNA topology via MukB, DNA gyrase, and Topo IV. These results demonstrate that SecA, SecY, and AcpP are involved in partitioning of the nucleoid. We note that a similar Par− phenotype is observed when the activity of DNA gyrase or Topo IV is inhibited (Wang, 2002), indicating a functional interaction among SecA, SecY, and AcpP with DNA gyrase and/or Topo IV.

Inhibition of the ATPase activity of SecA by 1 mM sodium azide caused a defect in proper cellular positioning of oriC and the Par− phenotype. Sodium azide at 1 mM neither significantly inhibited initiation nor ongoing chromosome replication (at least for 1 hour); the separation and bidirectional migration of two
bidirectional-replication forks to cell quarter positions from mid-cell (Hiraga et al., 2000; Onogi et al., 2002) were also not affected (Fig. S2). The cells with the wild-type \textit{secA} allele grew exponentially at least for 2 hours after the addition of 1 mM sodium azide (Fig. 1d). Thus, the chemical affected cell growth after a long time. The Par$^-$ phenotype observed in these \textit{secA}, \textit{secY}, and \textit{acpP} mutants at the nonpermissive temperatures may be due to a defect in decatenation of sister chromosomes, as supported by the two-dimensional gel electrophoresis of plasmid DNA (Fig. 3).

The \textit{secA51} (Ts) mutation caused both catenation and a reduction in the supercoiling of plasmids at the non-permissive temperature. This result suggests that the wild-type SecA protein is involved in the control of decatenation and superhelicity of plasmid DNA molecules. We postulate that the effects of SecA deficiency on the superhelicity of the plasmids and the partitioning of nucleoids are achieved via the functions of MukB, DNA gyrase, and possibly Topo IV. DNA gyrase has been suggested to decatenate circular DNA molecules only in
the presence of Topo IV *in vivo* (Zechiedrich & Cozzarelli, 1995). MukB likely forms DNA topology suitable for decatenation, and the absence of *mukB* results in DNA decatenation defects, possibly through this mechanism. In the Δ*mukB* mutant, superhelicity was still apparently normal, which may be due to weaker activities of both DNA gyrase (promoting negative superhelicity) and Topo IV (relaxing superhelicity). This is also consistent with the result that the secA51 single mutant at the non-permissive temperature, regardless of the presence of novobiocin (Fig. 3b, b’), exhibited plasmids with the same topology as the Δ*mukB* single mutant with novobiocin (Fig. 3e, e’) or the double secA51 and Δ*mukB* double mutant both in the presence and absence of novobiocin (Fig. 3f, f’, g, g’). SecA may be involved in the subcellular physiological regulation of MukB, DNA gyrase, and possibly Topo IV.

Inactivation of SecA may disrupt the translocation of membrane transporter proteins, resulting in abnormal intracellular physiology such as accumulation of membrane protein precursors or abnormal ionic conditions in which MukB, DNA
gyrase, and Topo IV are inactivated. However, we speculate that this hypothesis is unlikely according to the following observations. First, as mentioned later, our findings are supported by protein-protein interactions. Second, the secA204 mutant has a defect in the proper positioning of oriC foci, whereas membrane protein translocation activity is normal (Fig. 2f). The secA204 mutant may be a mutant of oriC positioning although the translocation activity is normal. Note that the effect of the secA204 mutation on oriC positioning is not due to inhibition of SecA ATPase activity (Fig. 2f, Oliver et al., 1990). We suggest that the altered SecA protein in the secA204 mutant may fail to interact with unknown proteins that are involved in the mechanism of oriC positioning such as the migS-dependent oriC positioning mechanism (Yamaichi & Niki, 2004). Certainly, additional effects on chromosome partitioning in secA may exist that are more important than oriC positioning. A comparison of the severe defects in secA51 mutants and the mild defect in secA204 mutants suggests the possible involvement of the essential AcpP.
Our current findings are further supported by protein-protein interactions, suggesting the existence of a chromosome partitioning protein network centralized by SecA. Chromosome partitioning proteins such as MukB, Topo IV, and DNA gyrase appear to physically interact with SecA, SecY, and AcpP, among approximately 4300 proteins in *E. coli* (Butland et al., 2005). The result of the large-scale protein-protein interaction network suggests the presence of a sub-network of Topo IV-MukB-AcpP-SecA, suggesting a possible functional link among these proteins. In addition, AcpP copurifies with MukB (Niki et al., 1992). Furthermore, SeqA (*Sequestration A*) protein, which binds to hemimethylated nascent DNA segments (Hiraga, 2000), interacts with ParC (Kang et al., 2003). Finally, stimulation of Topo IV activity by MukB and execution of MukB activity through Topo IV-MukB interactions have been shown in previous studies (Hayama & Marians, 2010; Li et al., 2010; Hayama et al., 2013). Fig. 4 shows a schematic of the physical interactions among these proteins and their relationships to the mechanism of chromosome partitioning, chromosome structural maintenance, and oriC positioning.
Our recent results (associated, submitted manuscript) suggest that this SecA-mediated mechanism acting together with DNA binding proteins is an important candidate for molecular ‘tethering’ during segregation of sister chromosomes and show the role of bacteria in the emerging field that unifies physics and biology on a micrometer scale.
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and seqA phenotypes might arise from their opposing influences on the

control genes affect chromosome superhelicity. EMBO Rep 1, RC494-RC499.

positioning of oriC on the Escherichia coli chromosome. EMBO J 23, 221-233.

genes, mukE and mukF, involved in chromosome partitioning in Escherichia coli.


**FIGURE LEGENDS**

Fig. 1. Sodium azide, secA, and secY mutations cause a chromosome partitioning defect. Cells were stained with DAPI. Arrowheads indicate elongated cells with a large nucleoid(s). White and black arrowheads indicate the cells in which Par^- phenotype were observed. (a) Temperature-sensitive secA51 (Ts) mutant (MM52) cells in medium L. A permissive temperature of 30°C and restrictive temperature of 42°C were used. (b) Cold-sensitive secY39 (Cs) mutant (AD208) cells in medium L. A permissive temperature of 37°C and restrictive temperature of 22°C were used. (c) Temperature-sensitive acpP D38V (Ts) mutant (NRD29) cells in RB medium. A permissive temperature of 30°C and restrictive temperature of 42°C were used. (d) IL05 (pLAU53) or isogenic secA204 mutant (MQ597) cells were treated with (open circles) or without (solid circles) 1 mM sodium azide at 37°C for 6 hours in medium L. Relative turbidity of cells is shown. (e) Cells of IL05 (pLAU53) or MQ597 (IL05 secA204) growing in medium C at 30°C were treated with or without 1 mM sodium azide for 3 hours. (f) Cells of IL05
(pLAU53) growing in medium L at 30°C were treated with or without 1 mM sodium azide for 3 hours.

Fig. 2. Sodium azide disrupts positioning of oriC. (a) The proportions of each type of IL05 (pLAU53) cells after the addition of 1 mM sodium azide. Cell types indicated with green bars are ‘irregular type (polar-type)’, in which at least one oriC focus was located in a pole zone. (b) The definition of cell zones. Total cell length is defined as 1. (c) The proportion of ‘irregular type (polar-type)’ cells in IL05 (pLAU53). (d) The proportions of each type of azide-resistant secA204 mutant (MQ597) cells after the addition of sodium azide. (e) The proportion of ‘irregular type (polar-type)’ cells in the azide-resistant secA204 mutant (MQ597). (f) Gel autoradiography of pre-MBP and MBP in the wild-type secA strain IL05 (pLAU53) and the azide-resistant secA204 mutant (MQ597). Lane 1, IL05 (pLAU53). Lane 2, IL05 (pLAU53) with 1 mM sodium azide. Lane 3, azide-resistant secA204 mutant (MQ597). Lane 4, azide-resistant secA204 mutant (MQ597) with 1 mM sodium azide. Upper band: pre-MBP. Lower band: MBP.
IL05 (pLAU53) and the azide-resistant secA204 mutant (MQ597) were incubated with or without 1 mM sodium azide at 30°C for 10 min before labeling.

Fig. 3. Two-dimensional gel electrophoresis of pUC19 plasmids shows secA activity that is related to DNA topology via MukB, DNA gyrase, and/or Topo IV. (a) to (f) Photographs of two-dimensional gel electrophoresis. Black and white photo images were reversed in the figure. (a) Wild-type cells (MC4100) at 30°C. (b) secA51 (Ts) mutant cells (MM52) incubated at the non-permissive temperature of 42°C for 2 hours. (c) Wild-type cells (MC4100) treated with 100 µg/ml novobiocin for 6 hours. (d) ΔmukB mutant cells (MQ761). (e) ΔmukB mutant cells (MQ761) treated with 100 µg/ml novobiocin for 6 hours. (f) secA51 ΔmukB double mutant cells (MQ760) treated with 100 µg/ml novobiocin for 6 hours. (g) secA51 ΔmukB double mutant cells (MQ760) without novobiocin treatment. (h) parC1215 (Ts) mutant cells (EJ812) incubated at the non-permissive temperature of 42°C for 2 hours. (i) Wild-type cells (MC4100) at 42°C. (a’) to (i’) Schematic interpretations of photographs. w, sample wells. g,
contaminating genomic DNA segments. 1ccc, covalently closed circular monomers of the plasmid DNA. Dotted line, the gel position corresponding to closed circular monomers in the wild-type strain (a).

Fig. 4. Protein-protein interaction map of proteins described in this manuscript. Interactions were deduced from Niki et al. (1992), Kang et al. (2003), Natale et al. (2004), Butland et al. (2005), Hayama & Marians (2010), Li et al. (2010), and Hayama et al. (2013). The large-scale interactions based on Butland et al. (2005) are indicated as short broken lines, and all other known interactions are indicated as solid lines. Long broken line shows that SecA is involved in the oriC positioning mechanism.
Table 1. *Escherichia coli* strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype/description</th>
<th>Resistance</th>
<th>Reference/source</th>
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<tr>
<td>W208</td>
<td><em>thr-1 leuB6</em>(Am) <em>secA204 lacZ4 glnV44</em>(As) <em>rpsL8 thi-1</em></td>
<td>Sm-R, Az-R</td>
<td><em>E. coli</em> Genetic Stock Center</td>
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<td>IL05 (pLAU53)</td>
<td>AB1157 <em>thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tss-33 supE44</em></td>
<td>Ap-R, Km-R, Gm-R</td>
<td>Lau <em>et al.</em> (2003)</td>
</tr>
<tr>
<td></td>
<td><em>tetO</em>240 @ <em>ori1 lacO</em>240 @ <em>ter2</em> (pLAU53)</td>
<td></td>
<td></td>
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<tr>
<td>MQ597</td>
<td>IL05 (pLAU53) except <em>secA204</em></td>
<td>Ap-R, Km-R, Gm-R, Az-R</td>
<td>This work</td>
</tr>
<tr>
<td>MC4100</td>
<td><em>araD139 Δ(argF-lac)U169 rpsL150 relA1 fhsB5301 deoC1 ptsF25 rbsR</em></td>
<td>Sm-R</td>
<td>Ito <em>et al.</em> (1981)</td>
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<tr>
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<td>Sm-R</td>
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<tr>
<td>MQ736</td>
<td>MC4100 (pUC19)</td>
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<td>This work</td>
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<td></td>
<td>Strain</td>
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<td>MQ508</td>
<td>MM52 except (λ-)</td>
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<td>TYO</td>
<td>MC4100 except ompT::kan znd33::Tn10 rpsE</td>
<td>Km-R Tc-R</td>
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<tr>
<td>C600</td>
<td>thi thr leuB lacY tonA supE</td>
<td></td>
<td>Appleyard (1954)</td>
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<td>EJ812</td>
<td>C600 except parC1215(Ts)</td>
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<td>W3110</td>
<td>IN (rrnD-rrnE) 1 rph-1</td>
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<td>YK1100</td>
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<tr>
<td>PC2</td>
<td>leuB6 : thyA47 rpsL153 (str) deoC3 dnaT12(Ts) dnaC2(Ts)</td>
<td>Sm-R</td>
<td>Carl (1970)</td>
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<td>MQ756</td>
<td>PC2 except secA204</td>
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Fig. 1
Fig. 2

(a) Wild-type

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<td>1B</td>
<td>18.3</td>
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<td>1C</td>
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<td>2A</td>
<td>28.4</td>
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<td>2C</td>
<td>12.5</td>
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<tr>
<td>2D</td>
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<td>2F</td>
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<td>2G</td>
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<td>2H</td>
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<td>2I</td>
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(b) Cell position

(c) Number of irregular-type cells (%)

(d) secA204 mutant

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<td>1B</td>
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<td>1C</td>
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<td>2C</td>
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(f) Gel autoradiography

Fig. 2
Fig. 4

Secretory system

SecY

SecA

oriC positioning mechanism

AcpP

MukB

SeqA (sequestration)

ParC

ParE

Topo IV

DNA gyrase

GyrA

GyrB

Chromosome partitioning mechanism

Inner membrane

Cytoplasm
No significant effect of sodium azide on the separation and bidirectional migration of replication forks.

Ongoing replication forks during bidirectional replication of chromosomes are separated from each other and positioned to cell quarter positions during replication in some growth conditions (Hiraga et al., 2000; Onogi et al., 2002; Yamazoe et al. 2005; Adachi et al. 2008). Using strain PC2, which has a temperature-sensitive mutation of replication initiation in dnaC, we examined the effect of 1 mM sodium azide on the separation and bidirectional migration of replication forks under conditions in which only one round of replication was synchronously initiated and further initiations were inhibited.

After incubation at 42°C for 60 min, the cells were shifted back to 30°C for 7 min to initiate replication and then shifted again to 42°C to inhibit further initiations. Five minutes after the shift back to 30°C, most cells had one or two SeqA-positive foci, suggesting the occurrence of chromosomal replication initiation (Fig. S2a).
Approximately 80% of cells had one focus, and approximately 20% of cells had two foci. The latter cells may correspond to cells with separated replication forks. Cells with two foci increased to 60% after 25 min, showing a peak of cells with two foci. After 45 min, cells with two foci disappeared, and cells with one focus increased. These results were consistent with previous results. SeqA binds to a terminal region after replication termination via an unknown mechanism, even though this region has been fully methylated (Yamazoe et al., 2005).

When 1 mM sodium azide was added to a subculture at time 0 (shift back to 30°C), most cells had one SeqA-positive focus at 5 min (Fig. S2b), suggesting that almost all cells synchronously initiated chromosomal replication after the shift back to 30°C, similar to the culture without sodium azide. Cells with two foci increased to 60% after 30 min, which was slightly delayed compared with the control culture without sodium azide. These results indicate that the addition of sodium azide does not significantly perturb the separation of replication forks during replication.
We analyzed the DNA content in each cell using flow cytometry after long incubation in the presence of rifampicin and cephalexin. When rifampicin and cephalexin were added at time 0, the DNA content per cell was equivalent to one chromosome. On the other hand, when these drugs were added at 7 min, the DNA content per cell was equivalent to two chromosomes, both in cultures with and without sodium azide (data not shown). These results indicated that one round of chromosome replication occurred in the presence of sodium azide as in the cultures with and without sodium azide.

The azide-resistant secA204 derivative of PC2 (MQ756) produced results that were almost identical to the results in PC2 without sodium azide, regardless of the presence of sodium azide (data not shown).

REFERENCES


### Table S1. Plasmids used.

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<tr>
<th>Plasmid</th>
<th>Replicon</th>
<th>Relevant genotype/description</th>
<th>Resistance</th>
<th>Reference/source</th>
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<tr>
<td>pLAU53</td>
<td>pMB1</td>
<td>pBAD::lacI::ecfp::tetR::eyfp</td>
<td>Ap-R</td>
<td>Lau <em>et al.</em> (2003)</td>
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</table>

### REFERENCES


SUPPLEMENTARY FIGURE LEGENDS

Fig. S1. λ phage induction of the secA51 (Ts) mutant (MQ508) in various conditions. (a) Number of plaques of λ phage at 30°C. (b) Number of plaques of λ phage at 42°C. Solid circles, without mitomycin C (MMC). Open circles, with 1 µg/ml MMC. λ phage was not induced by a temperature shift to 42°C, although it was induced by the addition of MMC as a positive control.

Fig. S2. No effect of 1 mM sodium azide on the separation and bidirectional migration of replication forks. Cells of strain PC2 (temperature-sensitive initiation dnaC mutant) were treated with three temperature shifts to synchronously initiate one round of chromosome replication, but further initiations were blocked (Yamazoe et al., 2005). Cells were removed at intervals, fixed with 70% methanol, and immunostained for SeqA protein. (a) Control culture without sodium azide. (b) Sodium azide (1 mM) was added at time 0 (shift back to 30°C). Blue circles, cells with one SeqA-positive focus. Black
circles, cells with two foci. Open triangles, cells with three foci. Solid triangles, cells with four foci.
Fig. S1.
Fig. S2.