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

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10

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.

1 INTRODUCTION

2 The mechanisms of chromosome partitioning in bacterial cells are not known.

3 Currently, two general issues are important in addressing the problem (Fisher *et*

4 *al.*, 2013): (I) how the cell places sister chromosomes in distinct spaces, also

5 named ‘positioning’; (II) the nature of the energy that is required for the process.

6 In this paper, we mainly investigated (I), the nature of ‘positioning’, and describe

7 the underlying mechanisms involved in the general topology of chromosomes.

8
9 In *Escherichia coli*, the replication origin of chromosome (*oriC*) localizes at mid-

10 cell before the onset of replication, and then the replicated sister *oriC* copies

11 move to the quarter cellular positions after duplication. Similarly, MukB

12 (“*mukaku*” means anucleate in Japanese), which is a functional homologue of

13 SMC (Structural Maintenance of Chromosomes) that mediates chromosome

14 organization, localizes equidistantly along the long cell axis as foci in living cells

15 (Hiraga, 2000; Ohsumi *et al.*, 2001; Adachi *et al.*, 2008; for a review, see Nolivos

16 & Sherratt, 2013). In $\Delta mukB$ (*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 & Mariani, 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.

1
2 In *E. coli*, Sun & Margolin (2004) briefly described the Par⁻ phenotype in SecA
3 (Secretory A) conditional mutant cells, which they used to address the function of
4 FtsZ (tubulin homologue). We are interested in the underlying molecular
5 mechanism that causes the chromosome partitioning defect in the absence of
6 SecA. SecA mediates translocation of membrane proteins through the Sec pore
7 complex of the inner cell membrane, thus maintaining homeostatic conditions in
8 cells via the membrane transport machinery (Roboson & Collinson, 2006). SecY
9 is a component of the membrane protein translocation channel together with SecE
10 and SecG (Oliver *et al.*, 1990). SecY and SecB interact with SecA and stimulate
11 the ATPase activity of SecA (Miller *et al.*, 2002; Natale *et al.*, 2004). Therefore,
12 we also investigated the molecular link between SecA and proteins known to be
13 involved in chromosome partitioning such as MukB, DNA gyrase, and Topo IV.
14

1 In the present work, we demonstrate the roles of SecA_SecY (a component of the
2 membrane protein translocation channel) and also AcpP, which is an interacting
3 partner of SecA, in proper chromosome partitioning.

4
5 AcpP is involved in fatty acid biosynthesis. AcpP is the sole essential acyl carrier
6 protein in *E. coli* and is acylated during fatty acid biosynthesis as a carrier and
7 donor (De Lay & Cronan, 2006). However, AcpP is also a hub protein in *E. coli*
8 and interacts with MukB as detected with both co-purification (Niki *et al.*, 1992)
9 and high-throughput analysis (Butland *et al.*, 2005). AcpP also interacts with
10 proteins not related to lipid synthesis, such as DNA polymerase III, RNA
11 polymerase, and ribosomes, suggesting a variety of functions and the possibility
12 of a role in chromosome partitioning when working together with MukB. Finally,
13 we found functional epistasis of SecA over MukB, DNA gyrase, and Topo IV,
14 revealing a novel role for the membrane protein translocation machinery in
15 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 $\Delta mukB$ mutation were obtained with co-transduction with the kanamycin resistance gene from AZ5372 and were selected and purified with 30 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ L-

1 threonine, L-leucine, L(-)-proline, L-arginine, L-histidine, L-tryptophan, and 5
2 µg/ml vitamin B1. Ampicillin (50 µg/ml), kanamycin (30 µg/ml), and gentamycin
3 (10 µg/ml) were added to the cultures of strains when necessary. For strain IL05
4 (pLAU53), L-(+)-arabinose (0.01%), anhydrotetracycline (12 nM), and isopropyl
5 β-D-1-thiogalactopyranoside (0.5 mM) were added to the culture (Lau *et al.*,
6 2003).

7
8 **Fluorescence Microscopy.** Microscopy was performed as described in
9 Adachi *et al.* (2008). When necessary, cells were stained with DAPI (4',6-
10 diamidino-2-phenylindole) and observed according to the fluorescence-phase
11 combined method described by Hiraga *et al.* (1989).

12
13 **Protein translocation assay *in vivo*.** The protein translocation assay *in*
14 *vivo* was performed according to Oliver *et al.* (1990). Bacterial cultures were
15 grown at 30°C in M9 medium containing 0.5% maltose, 50 µg/ml L-threonine, L-
16 leucine, L(-)-proline, L-arginine, L-histidine, L-tryptophan, and 5 µg/ml vitamin

1 B1. Ampicillin (50 µg/ml), kanamycin (30 µg/ml), and gentamycin (10 µg/ml)
2 were added when necessary. In the mid-exponential phase, 1 mM sodium azide
3 was added when necessary and incubated for another 10 min. Then, 20 µCi of a
4 mixture of 73% L-[³⁵S]methionine and 22% L-[³⁵S]cysteine (>1000 Ci/mmol;
5 PerkinElmer, Inc., Waltham, MA) were added to 1 ml of the culture and
6 incubated for 5 min (30°C), followed by addition of an equal volume of ice-cold
7 10% trichloroacetic acid. Samples were incubated on ice for 15 min, centrifuged
8 for 45 min at 4°C, 15,000 × *g*, and washed with acetone. Then we performed
9 immunoprecipitation analysis as described (Kruse *et al.*, 2006). Samples were
10 suspended in 80 µl lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1%
11 Nonidet, and a cocktail of EDTA-free protease inhibitors; Nacalai Tesque, Inc.,
12 Kyoto, Japan), placed on ice for 20 min, sonicated, and cell debris was removed
13 by centrifugation at 15,000 × *g* for 30 min at 4°C. Cleared cell lysates were
14 incubated for 30 min at 4°C with pretreatment resin, and then the supernatants
15 were incubated for 4 hours at 4°C with mouse anti-maltose binding protein
16 (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

1 soaked for 3 hours in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) containing 25
2 µg/ml (~80 µM) chloroquine. Then, the second-dimension run was performed at
3 1 V/cm for 18 hours, resulting in higher mobility for DNA with less negative
4 supercoiling (Hinton *et al.*, 1992). Plasmid DNA was detected by staining with 1
5 µg/ml ethidium bromide after the electrophoresis.

6
7 **Immunofluorescence microscopy.** Indirect immunofluorescence
8 microscopy using SeqA antiserum and goat anti-rabbit IgG antiserum was
9 performed as described previously (Hiraga *et al.*, 1998). We used goat anti-rabbit
10 IgG antiserum conjugated to Cy3 (GE Healthcare, U.K. Ltd.) as the secondary
11 antibody.

12
13 **Run-off replication method to analyze the DNA content per cell**
14 **using flow cytometry.** This procedure was performed as described (Adachi *et*
15 *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 $\Delta mukB$ 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

1 of 1 mM sodium azide because an azide-resistant *secA204* mutant (MQ597) grew
2 exponentially in the presence of 1 mM sodium azide and grew as well as the
3 mutant in the absence of the chemical (Fig. 1d). On the other hand, the isogenic
4 strain with the wild-type *secA* allele (IL05/pLAU53) grew exponentially for 2
5 hours after the addition of sodium azide, and then the growth was gradually
6 inhibited (Fig. 1d). Furthermore, the nucleoid morphology observed in the mutant
7 grown in 1 mM sodium azide was entirely normal (99/99 control cells and 71/71
8 azide-treated cells; Fig. 1e). We believe that observed effects of sodium azide are
9 due only to inactivation of SecA ATPase activity, and not to other effects at this
10 low concentration. When IL05 (pLAU53) cells were incubated with 1 mM
11 sodium azide for 4 hours at 30°C in medium C (minimal medium with a few
12 supplementary amino acids), approximately 30% (from 4/602 at 0 hours to
13 178/626 at 4 hours) of cells showed the typical Par^- phenotype (Fig. 1e). The
14 proportion of elongated cells increased with further incubation. Similar results
15 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^- 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 λ phage (a response of SOS) was not observed in the λ -phage lysogenized wild-type *secA* strain (MQ504) in the presence of 1 mM sodium azide or in the λ -phage lysogenized *secA51* mutant (MQ508) at the non-permissive temperature (Fig. S1). As a positive control, mitomycin C (1 $\mu\text{g/ml}$), which causes DNA damage, was used to induce λ -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*

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

9
10 We used the IL05 (pLAU53) strain, which contains 240 repeats of the *tetO*
11 insertion near *oriC* that can be visualized as fluorescent foci by TetR-EYFP (Lau
12 *et al.*, 2003). Cells of this strain grew exponentially with a doubling time of 150
13 min in medium C at 30°C. Samples were taken at various intervals and observed
14 with a fluorescence microscope for *oriC* foci. According to Bates and Kleckner
15 (2005), the *oriC* locus localizes at a new pole-biased region at the beginning of
16 the normal cell cycle. The locus then moves to mid-cell, begins replicating, and

1 after a brief cohesion, the focus splits into two. One remains at mid-cell, and the
2 other moves towards the 1/4 position in length. Thereafter, the mid-cell focus
3 moves towards the 3/4 position, and each locus remains at its corresponding
4 position until cell division. We normalized the cell length and divided it into five
5 zones as shown in Fig. 2b. Then, the intracellular localization of *oriC* foci was
6 classified into 12 types as shown in Fig. 2a. Because *oriC* never localizes very
7 close to cell poles in the normal Bates and Kleckner scheme, these 12 types were
8 grouped into two groups, “irregular-type (polar-type)” (Types 1C, 2E, 2F, 2G, 2H,
9 & 2I) and “regular-type (nonpolar-type)” (the remaining types), depending on
10 whether at least one *oriC* focus was abnormally localized at a cell pole. Before
11 inhibition of SecA with sodium azide, we found fewer than 2% of irregular-type
12 cells in the sample. However, irregular-type cells increased to approximately 20%
13 1 hour after the addition of 1 mM sodium azide (Fig. 2a, c).

14
15 As shown in Figure 2d and e, in the azide-resistant *secA204* mutant (MQ597),
16 irregular-type cells were observed at high frequency (approximately 15%), even

1 in the absence of sodium azide. In the membrane translocation assay with MBP,
2 the precursor of MBP (pre-MBP), which was larger than the membrane-
3 translocated mature MBP lacking the signal peptide, was not observed in the
4 *secA204* mutant cells grown in either the absence or presence of 1 mM sodium
5 azide (Fig. 2f, lanes 3 and 4). This indicates that the mutant has normal
6 translocation activity for membrane proteins in both growth conditions. This is
7 consistent with previous results reported by Oliver *et al.* (1990) who showed that
8 the *secA204* mutation does not affect SecA ATPase activity or the translocation
9 activity for MBP *in vivo*. Thus, although the altered SecA protein in the mutant
10 has normal translocation activity for membrane proteins, the protein is defective
11 in its ability to affect *oriC* positioning.

13 **SecA affects the topology of plasmid DNA**

14 Topological regulation of nucleoids or chromosomal regions is considered to be
15 important for proper chromosome partitioning in bacteria because proteins
16 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 $\mu\text{g/ml}$ *in vivo* (Adachi & Hiraga, 2003). When cells of the wild-type strain (MC4100) were incubated in the presence of novobiocin (100 $\mu\text{g/ml}$) 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 $\Delta mukB$ 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 *mukB* mutation did not markedly

1 affect the superhelicity. However, many additional spots were observed that
2 corresponded to concatemers of plasmid DNA monomers, which moved slowly
3 in the first-dimension electrophoresis.

4
5 We speculate that the defects in the SecA function in plasmid topology are
6 synthetic effects due to defects in DNA gyrase and MukB. $\Delta mukB$ mutant cells
7 are hypersensitive to novobiocin (Weitao *et al.*, 1999; Onogi *et al.*, 2000).
8 Novobiocin at 100 $\mu\text{g/ml}$ is a lethal dose in $\Delta mukB$ mutant cells, but not in wild-
9 type cells (Adachi & Hiraga, 2003). This concentration of novobiocin inhibits
10 separation (decatenation) of sister chromosomes in the $\Delta mukB$ mutant. Therefore,
11 we used this concentration to further study the effect of novobiocin in lethality.
12 When $\Delta mukB$ mutant cells were treated with 100 $\mu\text{g/ml}$ novobiocin for 4 hours
13 followed by a shift up to the non-permissive temperature of 42°C for 2 hours,
14 covalently closed circular monomers moved rapidly in the second-dimension
15 electrophoresis (Fig. 3e, e'), similar to the *secA51* mutant at the non-permissive
16 temperature (Fig. 3b, b'). A large number and high amount of additional spots

1 corresponding to concatemers were also observed in this condition, the same as
2 the *secA51* mutant at the non-permissive temperature.

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

11
12 Topo IV is known to relax the superhelicity of plasmid DNA molecules and to
13 decatenate DNA concatemers. As another control, we examined the effect of
14 inactivation of Topo IV on plasmid topology. We used a temperature-sensitive
15 *parC1215* mutant (EJ812) with a mutation in the gene encoding the Topo IV
16 subunit ParC. When the *parC1215* mutant cells were incubated at the non-

1 permissive temperature of 42°C for 2 hours, covalently closed circular monomer
2 molecules from the mutant cells were detected as three spots that moved more
3 slowly (Fig. 3h, h') than covalently closed circular monomers from wild-type
4 cells (Fig. 3a, a') in the second-dimension electrophoresis as expected, suggesting
5 an increase in negative superhelicity at three different levels. The negative
6 superhelicity of the plasmids in the other spots was also increased, suggesting
7 inactivation of the relaxation activity of Topo IV. In this condition, the amount of
8 plasmid concatemer is less than 10% (Zechiedrich & Cozzarelli, 1995), indicating
9 that very few concatemers are visualized in the gel.

10
11 The temperature difference between 42°C and 30°C did not significantly affect
12 the pattern of the plasmid topology in the wild-type strain (Fig. 3i, i', a, a'),
13 indicating that the temperature variations used in this study did not affect DNA
14 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

1 bidirectional-replication forks to cell quarter positions from mid-cell (Hiraga *et*
2 *al.*, 2000; Onogi *et al.*, 2002) were also not affected (Fig. S2). The cells with the
3 wild-type *secA* allele grew exponentially at least for 2 hours after the addition of
4 1 mM sodium azide (Fig. 1d). Thus, the chemical affected cell growth after a long
5 time. The Par^- phenotype observed in these *secA*, *secY*, and *acpP* mutants at the
6 nonpermissive temperatures may be due to a defect in decatenation of sister
7 chromosomes, as supported by the two-dimensional gel electrophoresis of
8 plasmid DNA (Fig. 3).

9
10 The *secA51* (Ts) mutation caused both catenation and a reduction in the
11 supercoiling of plasmids at the non-permissive temperature. This result suggests
12 that the wild-type SecA protein is involved in the control of decatenation and
13 superhelicity of plasmid DNA molecules. We postulate that the effects of SecA
14 deficiency on the superhelicity of the plasmids and the partitioning of nucleoids
15 are achieved via the functions of MukB, DNA gyrase, and possibly Topo IV.
16 DNA gyrase has been suggested to decatenate circular DNA molecules only in

1 the presence of Topo IV *in vivo* (Zechiedrich & Cozzarelli, 1995). MukB likely
2 forms DNA topology suitable for decatenation, and the absence of *mukB* results
3 in DNA decatenation defects, possibly through this mechanism. In the *ΔmukB*
4 mutant, superhelicity was still apparently normal, which may be due to weaker
5 activities of both DNA gyrase (promoting negative superhelicity) and Topo IV
6 (relaxing superhelicity). This is also consistent with the result that the *secA51*
7 single mutant at the non-permissive temperature, regardless of the presence of
8 novobiocin (Fig. 3b, b'), exhibited plasmids with the same topology as the
9 *ΔmukB* single mutant with novobiocin (Fig. 3e, e') or the double *secA51* and
10 *ΔmukB* double mutant both in the presence and absence of novobiocin (Fig. 3f, f',
11 g, g'). SecA may be involved in the subcellular physiological regulation of MukB,
12 DNA gyrase, and possibly Topo IV.

13
14 Inactivation of SecA may disrupt the translocation of membrane transporter
15 proteins, resulting in abnormal intracellular physiology such as accumulation of
16 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.

1 Our current findings are further supported by protein-protein interactions,
2 suggesting the existence of a chromosome partitioning protein network
3 centralized by SecA. Chromosome partitioning proteins such as MukB, Topo IV,
4 and DNA gyrase appear to physically interact with SecA, SecY, and AcpP, among
5 approximately 4300 proteins in *E. coli* (Butland *et al.*, 2005). The result of the
6 large-scale protein-protein interaction network suggests the presence of a sub-
7 network of Topo IV-MukB-AcpP-SecA, suggesting a possible functional link
8 among these proteins. In addition, AcpP copurifies with MukB (Niki *et al.*, 1992).
9 Furthermore, SeqA (*Sequestration A*) protein, which binds to hemimethylated
10 nascent DNA segments (Hiraga, 2000), interacts with ParC (Kang *et al.*, 2003).
11 Finally, stimulation of Topo IV activity by MukB and execution of MukB activity
12 through Topo IV-MukB interactions have been shown in previous studies
13 (Hayama & Marians, 2010; Li *et al.*, 2010; Hayama *et al.*, 2013). Fig. 4 shows a
14 schematic of the physical interactions among these proteins and their
15 relationships to the mechanism of chromosome partitioning, chromosome
16 structural maintenance, and *oriC* positioning.

1

2 Our recent results (associated, submitted manuscript) suggest that this SecA-
3 mediated mechanism acting together with DNA binding proteins is an important
4 candidate for molecular ‘tethering’ during segregation of sister chromosomes and
5 show the role of bacteria in the emerging field that unifies physics and biology on
6 a micrometer scale.

7

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- 8

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 *acpPD38V* (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.

Strain	Relevant genotype/description	Resistance	Reference/source
W208	<i>thr-1 leuB6</i> (Am) <i>secA204 lacZ4 glnV44</i> (As) <i>rpsL8 thi-1</i>	Sm-R, Az-R	<i>E. coli</i> Genetic Stock Center
IL05 (pLAU53)	AB1157 <i>thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44</i> <i>tetO₂₄₀ @ ori1 lacO₂₄₀ @ ter2</i> (pLAU53)	Ap-R, Km-R, Gm-R	Lau <i>et al.</i> (2003)
MQ597	IL05 (pLAU53) except <i>secA204</i>	Ap-R, Km-R, Gm-R, Az-R	This work
MC4100	<i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Sm-R	Ito <i>et al.</i> (1981)
MM52	MC4100 except <i>secA51</i> (Ts)	Sm-R	Oliver & Beckwith (1981)
MQ736	MC4100 (pUC19)	Ap-R, Sm-R	This work

MQ734	MM52 (pUC19)	Ap-R, Sm-R	This work
MQ761	MC4100 $\Delta mukB::kan$ (pUC19)	Ap-R, Sm-R	This work
MQ760	MM52 $\Delta mukB::kan$ (pUC19)	Ap-R, Sm-R	This work
MQ508	MM52 except (λ^+)	Sm-R	This work
TYO	MC4100 except <i>ompT::kan znd33::Tn10 rpsE</i>	Km-R Tc-R	Akiyama & Ito (1990)
AD208	TYO except <i>secY39</i> (Cs)	Km-R Tc-R	Baba <i>et al.</i> (1990)
NRD52	MG1655 except <i>$\Delta fabF::cat$</i>	Cm-R	De Lay & Cronan (2006)
NRD29	NRD52 except <i>acpP</i> D38V	Cm-R	De Lay & Cronan (2006)
C600	<i>thi thr leuB lacY tonA supE</i>		Appleyard (1954)
EJ812	C600 except <i>parC1215</i> (Ts)		Kato <i>et al.</i> (1988)
MQ738	C600 (pUC19)	Ap-R	This work

MQ739	EJ812 (pUC19)	Ap-R	This work
W3110	IN (<i>rrnD-rrnE</i>) 1 <i>rph-1</i>		Hill & Harnish (1988)
YK1100	W3110 except <i>trpC9941</i>		Yamanaka <i>et al.</i> (1996)
AZ5372	YK1100 except $\Delta mukB::kan$	Km-R	Yamanaka <i>et al.</i> (1996)
MQ504	YK1100 except (λ^+)		This work
PC2	<i>leuB6</i> λ^- <i>thyA47 rpsL153 (str) deoC3 dnaT12(Ts) dnaC2(Ts)</i>	Sm-R	Carl (1970)
MQ756	PC2 except <i>secA204</i>	Az-R, Sm-R	This work

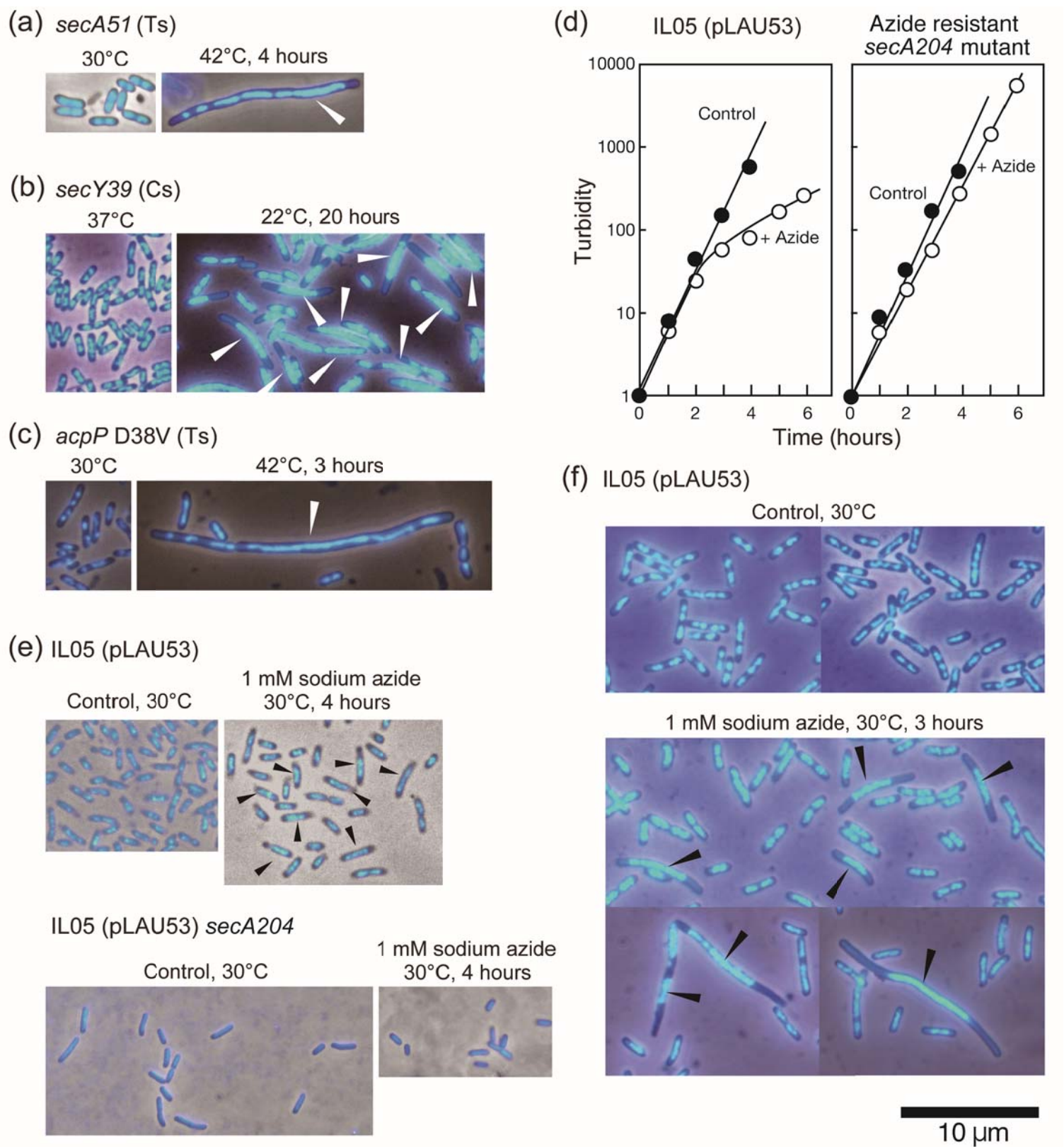


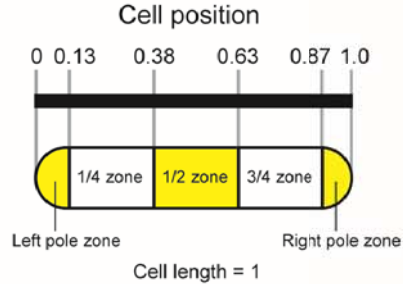
Fig. 1

(a) Wild-type

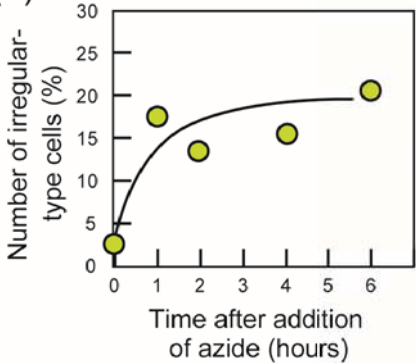
Cell type	Number of cells (%)				
	0 h n = 464	1 h n = 525	2 h n = 515	4 h n = 815	6 h n = 569
1A	31.7	23.6	27.2	36.1	24.1
1B	18.3	26.9	28.5	22.1	15.5
1C	1.1	6.3	4.9	3.4	1.4
2A	28.4	26.3	23.3	17.1	12.1
2B	5.4	1.1	0.4	2.6	4.7
2C	12.5	4.6	5.6	6.1	20.6
2D	1.3	0	1.4	0.7	2.3
2E	1.1	3.2	3.7	9.3	13.7
2F	0.2	6.9	3.5	1.2	2.1
2G	0	0.4	0.8	1.2	2.5
2H	0	0.8	0.8	0	0.4
2I	0	0	0	0.1	0.7

Irregular types h, hours

(b)



(c)

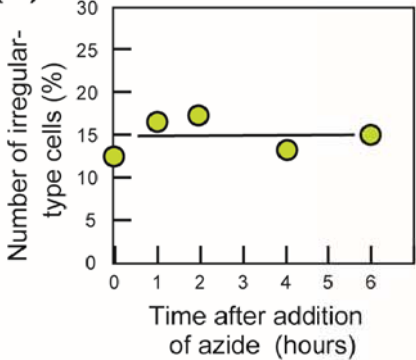


(d) *secA204* mutant

Cell type	Number of cells (%)				
	0 h n = 695	1 h n = 1332	2 h n = 812	4 h n = 914	6 h n = 1127
1A	34.2	33.2	32.1	30.6	34.2
1B	10.5	7.9	12.1	10.8	10.0
1C	2.9	5.0	3.9	1.2	3.0
2A	23.5	29.2	20.0	23.4	22.0
2B	5.2	4.7	3.2	4.9	5.8
2C	13.4	7.4	11.8	13.5	10.0
2D	0.7	1.8	3.8	3.0	3.0
2E	6.2	7.8	10.8	10.3	9.8
2F	0.09	0.5	0.6	0.7	0.4
2G	2.2	2.3	0.7	1.3	1.8
2H	0.3	0.2	0	0	0
2I	0.1	0.2	0.9	0.3	0.1

Irregular types h, hours

(e)



(f) Gel autoradiography

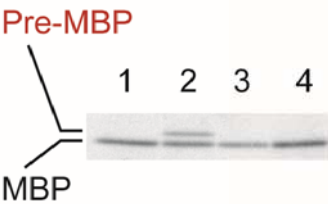


Fig. 2

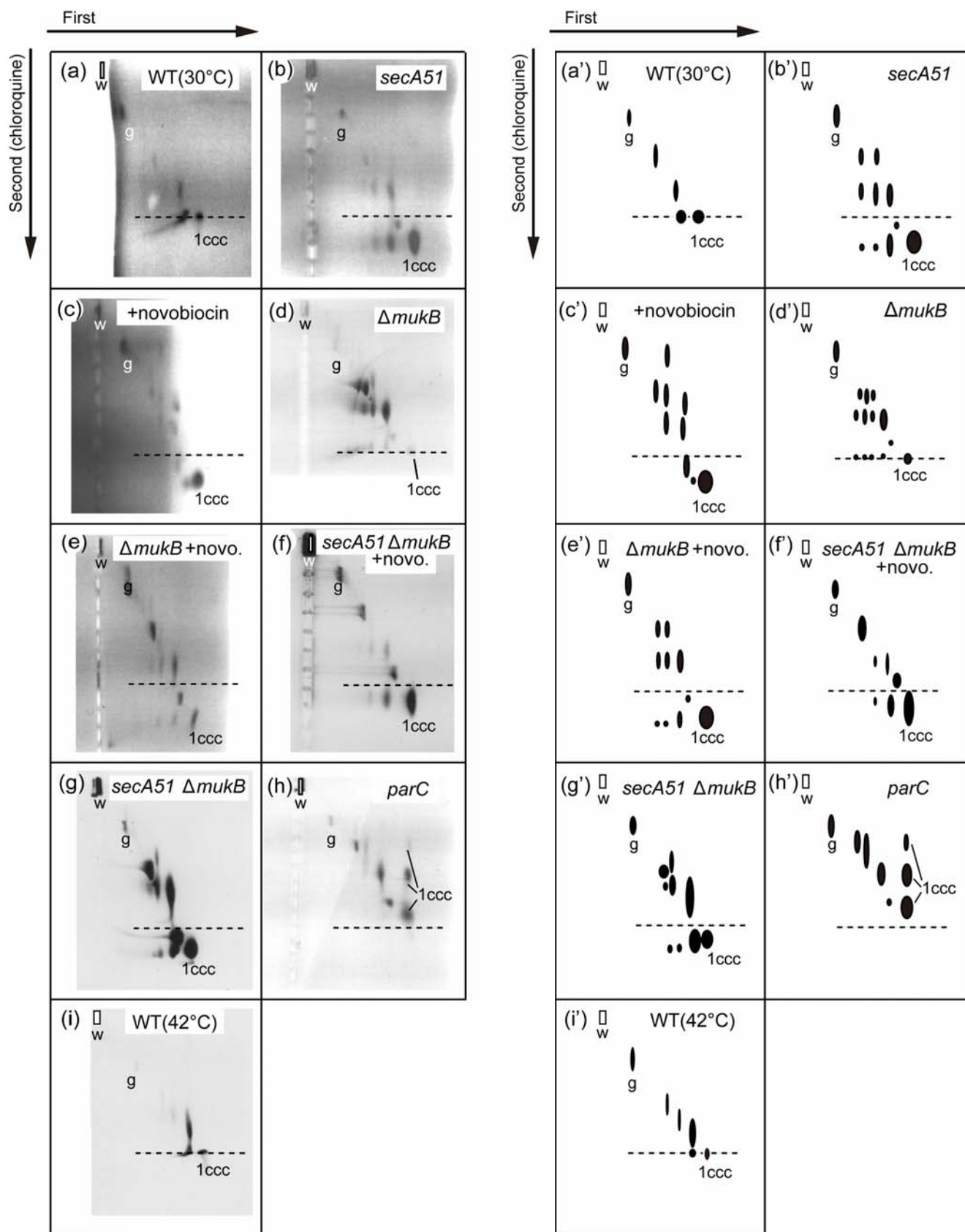


Fig. 3

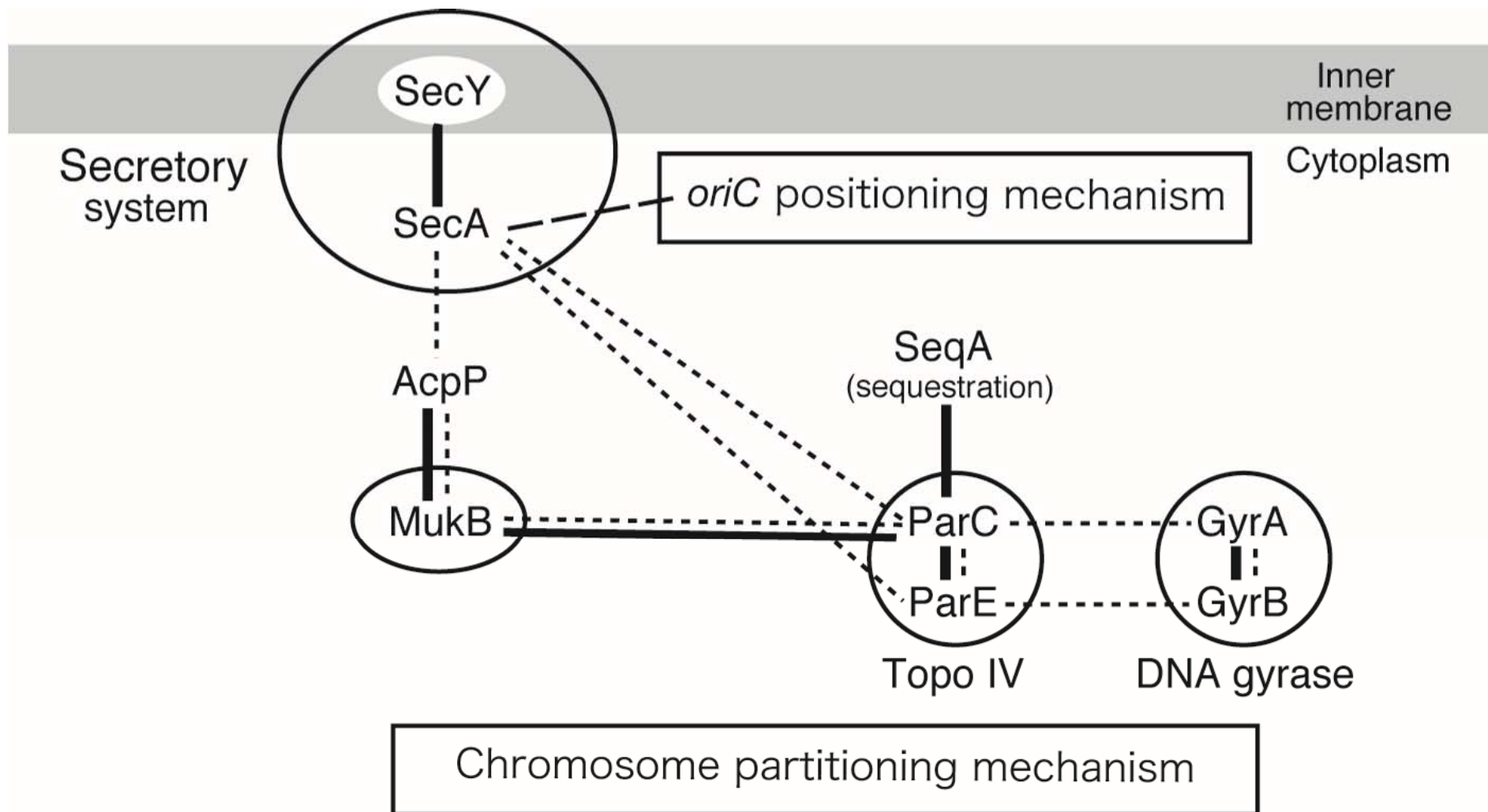


Fig. 4

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 cephalixin. When rifampicin and cephalixin 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).

1

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8 binding of SeqA protein to nascent DNA segments at replication forks in
9 synchronized cultures of *Escherichia coli*. *Mol Microbiol* **55**, 289-298.

Table S1. Plasmids used.

Plasmid	Replicon	Relevant genotype/description	Resistance	Reference/source
pUC19	pMB1		Ap-R	Yanisch-Perron <i>et al.</i> (1985)
pLAU53	pMB1	pBAD::lacI::ecfp::tetR::eyfp	Ap-R	Lau <i>et al.</i> (2003)

REFERENCES

Lau, I.F., Filipe, S.R., Søballe, B., Økstad, O.A., Barre, F.X. & Sherratt, D.J. (2003) Spatial and temporal organization of replicating *Escherichia coli* chromosomes. *Mol Microbiol* **49**, 731-743.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

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 μ g/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.

1

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

1 circles, cells with two foci. Open triangles, cells with three foci. Solid triangles,

2 cells with four foci.

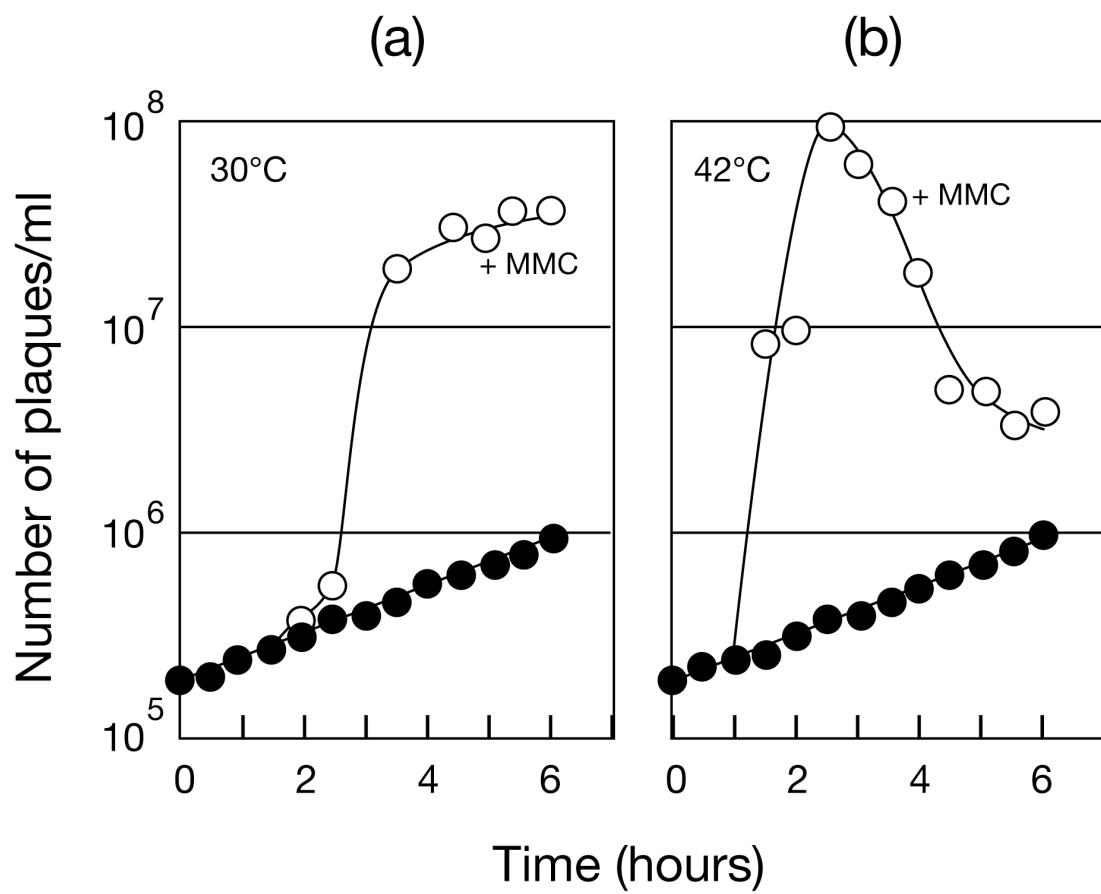


Fig. S1.

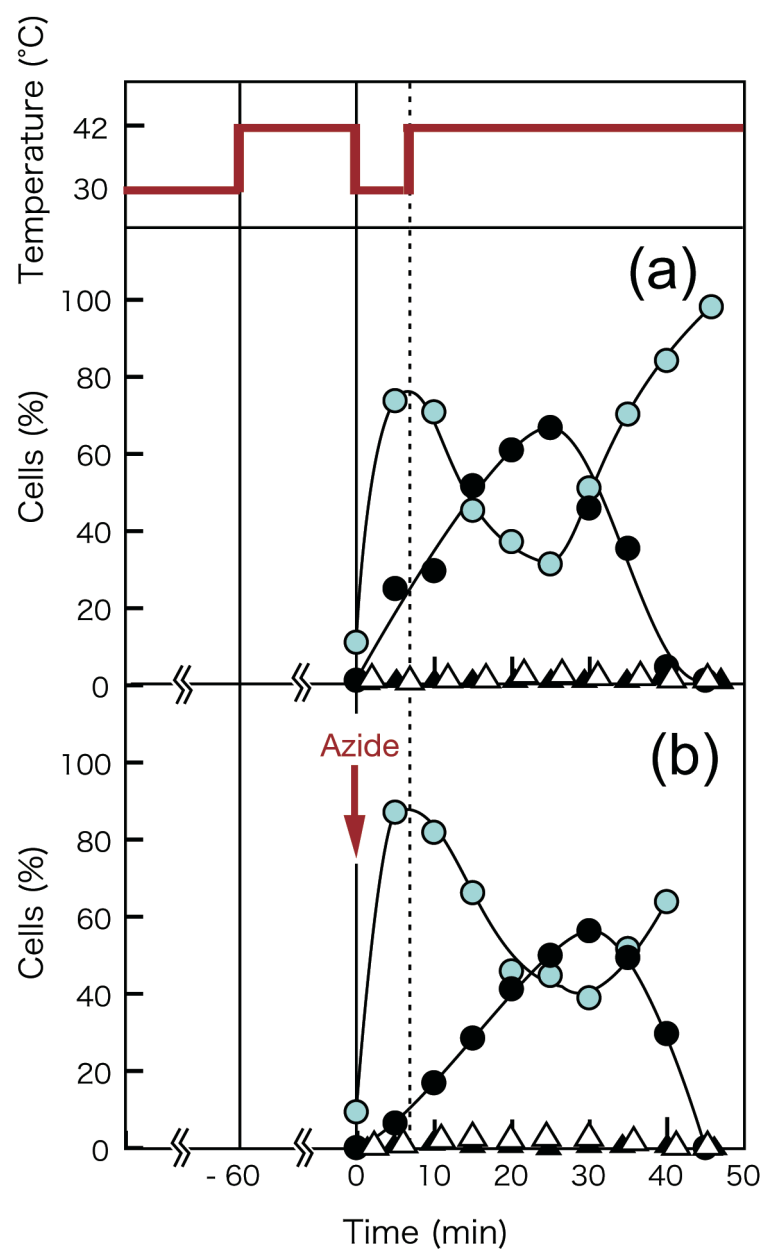


Fig. S2.