Profiling a single-stranded DNA region within an rDNA segment that affects the loading of bacterial condensin

**Highlights**
- A direct bisulfite treatment to a cell can modify cytosine at a ssDNA segment
- The CT conversion rate may reflect the ssDNA formation in the cell
- Transcription activity enhances the CT conversion rate
- The ssDNA formation near the promoter is involved in the loading of Smc-ScpAB

**Abstract**

Yano et al., *iScience* 25, 105504
December 22, 2022 © 2022
The Author(s).
https://doi.org/10.1016/j.isci.2022.105504
Profiling a single-stranded DNA region within an rDNA segment that affects the loading of bacterial condensin

Koichi Yano,1 Hideki Noguchi,2,3 and Hironori Niki4,5,*

SUMMARY

Bacterial condensin preferentially loads onto single-stranded DNA (ssDNA) in vitro and onto rDNA in vivo to support proper chromosome compaction. Thus, the actively transcribing rDNA would provide the ssDNA region for the loading of bacterial condensin. We attempted to detect the ssDNA region in situ. Non-denaturing sodium bisulfite treatment catalyzed the conversion of cytosines to thymines (CT-conversion) at the melted DNA of a genome. Using next-generation sequencing, we generated an average of 11,000 reads covering each cytosine on the rDNA segment. In principle, the CT-conversion rate is an accurate guide to detect ssDNA segment. We detected multiple ssDNA segments throughout the rDNA. The deletion mutations of the rDNA hindered the ssDNA formation at the 100-500 bp segment downstream of the promoter. These data support the idea that the ssDNA segment plays a crucial role as the condensin-loading site and suggest the mechanism of condensin loading onto rDNA.

INTRODUCTION

Proper organization of chromosomal DNA is crucial for the segregation of replicated chromosomes into daughter cells. The structural maintenance of chromosomes (SMC) proteins are major players in chromosomal DNA organization in prokaryotes and eukaryotes.1–5 The SMC proteins form the core of the two distinct complexes, cohesin, and condensin, that manage chromosome cohesion and condensation. A two-armed structure of the SMC dimer forms a ring that holds DNA topologically. The topological DNA-binding activity is regulated by non-SMC subunits that interact with an ATP-binding cassette (ABC)-like domain at the distal end of each arm. In addition, the SMC proteins bind to not only double-stranded DNA (dsDNA), but also single-stranded DNA (ssDNA). The physiological significance of the ssDNA-binding activity is currently not clear. However, several reports indicate that the ssDNA binding of the SMC proteins contributes to topological DNA binding on genomic DNA.6–12

The Bacillus subtilis Smc-ScpAB complex consists of the core of the SMC protein Smc and the non-SMC subunits ScpA/B and functions as a bacterial condensin.13,14 The Smc protein specifically accumulates at parS which is the Spo0J binding site on the B. subtilis chromosome.15 The Spo0J-parS facilitates the loading of the Smc-ScpAB complex on the chromosome to support efficient chromosome segregation by organizing the chromosome of B. subtilis.15,16 Deficiency of Spo0J, deficiency of parS, or both cause moderate deterioration of chromosome segregation, whereas deficiency of Smc severely prevents chromosome partitioning. The contrast indicates that another loading pathway of the Smc-ScpAB complex must work independently. In addition to accumulating at the Spo0J binding sites, Smc is enriched at the highly transcribed genes containing rDNA.15 Deficiencies of both rDNA and Spo0J cause aberrant nucleoid separation, which is similar to that of the smc deletion mutant.17 Thus, rDNA may also be a loading site for the Smc-ScpAB complex to perfectly organize the chromosome of B. subtilis.

Moreover, a plasmid harboring a full-length rDNA segment is effectively co-immunoprecipitated with the Smc-ScpAB complex when rDNA is actively transcribed.17 The highly transcribed rDNA would interact with the Smc-ScpAB complex. Although transcription is still highly activated, deletions of the rDNA segment on plasmids hinder efficiencies of co-immunoprecipitation of the plasmid DNA with the Smc-ScpAB complex. The full-length rDNA encodes 16, 23, 5S, and tRNA. These transcribed RNAs of the full-length rDNA...
segment form a higher-order structure. In particular, the ssDNA formation coupled with transcription or R-loop might be a leading cause of the interaction of the bacterial condensin with the plasmid DNA because the Escherichia coli condensin MukB topologically binds ssDNA preferentially rather than double-stranded DNA (dsDNA). Thus, it is conceivable that a higher-order structure of the transcripts and template DNA strand or R-loop may be involved in the topological loading of bacterial condensin.

Chromosomal DNA is ordinarily maintained as dsDNA and is partially melted as ssDNA during DNA replication and active transcription. It is conventionally considered that the ssDNA formation is detrimental to the maintenance of chromosome integrity. However, recent studies indicate that non-B DNA structures play physiological roles, including roles in transcription regulation, absorption of torsional stress, and DNA replication inhibition. In addition, dsDNA locally “breathes” to melt one to several base pairs and is used for the homology probing during homologous recombination. Several methods have been used to detect ssDNA, such as probing with an anti-DNA-RNA hybrid antibody against the R-loop and nuclease mapping for the ssDNA segments. In addition, bisulfite genomic sequencing, a gold-standard method to detect DNA methylation, also helps determine the ssDNA segments in genomic DNA. Bisulfite selectively deaminates cytosine residues of denatured DNA and catalyzes the conversion of unpaired cytosines to uracils.

**RESULTS**

*B. subtilis* condensin binds ssDNA. Moreover, *B. subtilis* condensin interacts with rDNA in vivo, which would work to support proper chromosome compaction. Based on those reports, we hypothesized that the rDNA provides the ssDNA region for the topological binding of the Smc-ScpAB complex. Therefore, we attempted to detect the ssDNA region on the rDNA segment in a living bacterial cell. Using next-generation sequencing (NGS), we generated 11,000 reads, on average, that cover each cytosine on the PCR-amplified rDNA segment to obtain the precise CT conversion rate. An outline of the non-denaturing sodium bisulfite sequencing is shown in Figure S1. The results showed that the CT conversion rates were higher than the frequency of error generation by NGS. Therefore, we expected that an increment in the CT conversion rate would reflect a trend toward ssDNA accumulation at a given site within the rDNA segment.

**In situ detection of the single-stranded DNA region in rDNA on the chromosome**

The wild-type cell of *B. subtilis* has ten copies of rDNAs. By contrast, the “2 rrn strain” has only two copies of the rRNA gene due to deletions in the other eight rDNAs. The specific rDNA segment of the 2 rrn strain was easily amplified by PCR. The 2 rrn strain exhibited slight defects in cell growth and had nucleoids that were separated and constricted in a dumbbell shape, as seen in the wild-type cell. And we carried out the co-immunoprecipitation of plasmids harboring a series of the rDNA segments with the Smc-ScpAB complex. Then we used a series of the 2 rrn strains harboring the deletion mutation rDNA segments to detect the ssDNA region on the rDNA segment (Figure S2).

First, we analyzed the CT conversion rates of the wild-type *rrn* gene of the 2 rrn mutant without the sodium bisulfite treatment. We determined the CT conversion rates of each cytosine at the top and the bottom strand in the 5′ half of the *rrn* gene. The CT conversion rates of the top strand were similar to those of the bottom strand (Figure 1A). We performed a sliding window analysis with the window size fixed at 64 bp and moving away from the promoter region of *rrn* in one base step (Figure 1B). The average CT conversion rate of each window was mapped on the *rrn* gene to compensate for the irregular cytosine distributions in the gene. There were no characteristic profiles of the CT conversion rates at either strand. The average CT conversion rate was 0.16% (Table S1). When cells were not exposed to sodium bisulfite, the low CT conversion rates were well consistent with the error rate caused by the misreading of NGS (0.1-0.3%).

By contrast, the top and bottom strand CT conversion rates in the 5′ half of the *rrn* gene were about 40-fold increased when cells were exposed to sodium bisulfite (Table S1). The average CT conversion rate was 6.89%. The CT conversion rates fluctuated dramatically at a single-nucleotide resolution throughout the
The lowest rate was 0.81%, and the highest rate was 21.5% (Figure 1C). We further analyzed the CT conversion rates of the 3' half of the rrnI gene and then characterized the CT conversion rates throughout the rrnI gene. Similar results were obtained (Table S1 and Figure 1C). The CT conversion rates fluctuated dramatically throughout the rrnI gene. The average CT conversion rate of the rrnI gene was 6.77%, and the highest rate was 25.6%. The nucleotide positions corresponding to the higher CT conversion rates (>15%) on the top and the bottom strand are listed with the neighbor sequences in Tables S2 and S3. In addition, nucleotides with higher CT conversion rates were simultaneously found on both the top and the bottom strand of the DNA segment (Figures S3 and S4). The nucleotides with higher CT conversion rates were positioned within DNA segments with relatively higher GC content, except for bp position 669 at the top strand.
which was located at a DNA segment with relatively high AT content (Table S2). Thus, the higher CT conversion frequencies in the *rrnI* gene might not be caused by high AT content at the DNA segment.

**Relationship between the CT conversion rates and the C content**

The sliding window analysis showed that the CT conversion rates fluctuated dramatically throughout the whole *rrnI* gene. We conjectured that the cytosine content within the sliding window affected the fluctuation of the CT conversion rates because the cytosine content within the window size used (64 bp) is not constant in the *rrnI* gene (Figure 1D). We analyzed the correlation coefficient between the CT conversion rates and the cytosine contents (Figure 2A). Weak positive correlations were observed in the top and the bottom strand. The correlation coefficients were 0.41 and 0.53, respectively.

However, several segments were plotted apart from the main correlation lines. Indeed, some windows showed that the CT conversion rates were relatively high even though the cytosine content (C content) was low. Conversely, other windows showed that the CT conversion rates were relatively low even though the cytosine content was high. We selected the sliding windows with the higher and lower CT conversion rates.
rates (more and less than 1SD, respectively) from Figure 2A and plotted them on the rml gene map along with the CT conversion rates (Figures 2B, 5S and 56, Tables S4–S6). We confirmed that the CT conversion rates at the specific segments of the rml gene increased independently of the cytosine content. These results suggest that the rml gene contained the DNA segments that tend to form ssDNA.

**Relation between CT conversion and transcription at the rDNA**

Next, we examined the effect of active transcription on the CT conversion rates. We analyzed the CT conversion rates of the rmlΔp gene in which the promoter region was deleted (YAN12675; Figure 3A). The transcriptional activity of the rmlΔp gene was reduced by 99% compared with that of the wild-type rml gene. However, a remarkable CT conversion of rmlΔp was detected in the rmlΔp gene. Figure 3 shows the profiles of the CT conversion rates of the single nucleotides and the sliding window analysis. The whole profiles were strikingly similar in appearance to the profiles of the wild-type rml gene. However, we considered that the marked decline in transcription activity caused the differences in the CT conversion rates between the wild-type and the rmlΔp. We then subtracted the rmlΔp CT conversion rates from the wild-type CT conversion rates for further analysis (Figure 3C). We found that, on the whole, the wild-type CT conversion rates were higher than the rmlΔp conversion rates. The transcription activity from the rml promoter does not directly affect DNA melting in the segment upstream of the rml promoter. Certainly, the CT conversion rate differences between the wild-type and the rmlΔp were slight at the segment upstream of the rml promoter. The average difference in the CT conversion rate at the promoter’s upstream segment was 0.52% (Table S7). On the other hand, the average difference in the CT conversion rate was 2.06 percentage points in the promoter’s downstream segment. Moreover, several segments showed differences of more than 4 percentage points in the CT conversion rate.

Notably, the differences in the CT conversion rates were particularly large in the top strand, i.e., the coding DNA strand. On the other hand, the differences in the CT conversion rates in the bottom strand, i.e., the template DNA strand, were mainly found at the former part of the rml gene but not at the latter part.

Next, we subtracted the CT conversion rates of the top strand from those of the bottom strand Figure 3D). The degree of influence caused by reducing the transcription activity was substantial in the top strand of the rml gene, and the influence was especially remarkable at the latter part of the top strand. The two peaks of the biased CT conversion rates at the segment 100 to 500 bp downstream of the promoter indicate that the ssDNA formation was affected by the reduction in transcription activity.

**The CT conversion of the rmlΔSS mutant**

We carried out a further analysis to specify the portion of the ssDNA region within the rml gene that affects the interaction of Smc-ScpAB bacterial condensin. We analyzed the CT conversion rate of the rmlΔSS gene (YAN12698), in which the 51 bp segment of the 5S rRNA coding region (116 bp) was deleted (Figure 4A). Although the mutated rmlΔSS gene results in a defect in the interaction of the Smc-ScpAB, it is actively transcribed. The whole profiles of the CT conversion of rmlΔSS shown by both the single nucleotides and the sliding window analysis were strikingly similar in appearance to those of the wild-type rml gene (Figures 4A and 4B). As in the analysis of rmlΔp, the CT conversion rate of the rmlΔSS gene was subtracted from that of the wild-type rml. As a result, we found marked differences in the CT conversion rates in the segment 100 to 500 bp downstream of the promoter but not in the other segments (Figure 4C). The CT conversion rates within the segment 100 to 500 bp downstream of the promoter were higher than the average CT conversion rate of the rml segment. Similar results were obtained from the bottom strand.

We subtracted the CT conversion rates of the bottom strand from those of the top strand (Figure 4D). There were two peaks in the CT conversion rates of the top strand in the segment 100 to 500 bp downstream of the promoter, as shown in Figure 4D. The deletion of the 51 bp segment at the 5S rRNA coding region specifically affected the CT conversion rates of the top strand. Thus, the efficiency of ssDNA formation within the rmlΔSS gene was reduced only at the downstream segment of the promoter.

**The CT conversion of an rDNA mutant defective in the interaction of the Smc-ScpAB complex**

Next, we analyzed other deletion mutants of the rml gene in the 2 rrn strain (Figure S2). These mutants of the rml gene maintained active transcription but lost their interaction with the Smc-ScpAB complex. The whole profiles of the CT conversion of the mutated rml genes are shown in Figures 5 and S7. As shown by the sliding window analyses of rmlΔ23′-SS and rmlΔ23-SS, the whole profiles of the CT conversion of
the remaining segments were similar in appearance to those of the wild-type \( rnl \) gene (Figure 1D). We found that the CT conversion rates were affected in the segment 100 to 500 bp downstream of the promoter when we subtracted the CT conversions of \( rnl^{D23-5S} \) from the wild-type or the CT conversions of \( rnl^{D23-5S} \) from the wild-type. Other deletion mutants of the \( rnl \) gene lost the region containing the segment 100 to 500 bp downstream of the promoter. The remaining segment was similar to that of the wild-type \( rnl \) gene, and the differences were very small between the wild-type and the \( rnl \) mutant genes.

Figure 3. The CT conversion rates of the \( rnl^{\Delta p} \) segment
(A) The CT conversion rates at each cytosine of the \( rnl^{\Delta p} \) segment with sodium bisulfite treatment. The CT conversion rates of the top (red) and the bottom (green) strand of the \( rnl^{\Delta p} \) segment are represented. The deleted region is indicated with a gray box in the \( rnl \) gene map.
(B) The CT conversion rates of the \( rnl^{\Delta p} \) segment with sodium bisulfite treatment are represented as the results of a sliding window analysis (window size of 64 bp).
(C) The differences in the CT conversion rates between the \( rnl^{\Delta p} \) segment and the wild-type \( rnl \) segment. The left vertical line shows the differences in the CT conversion rates for the top strand, and the right vertical axis shows those for the bottom strand.
(D) The differences in the CT conversion rates between the top and the bottom strand as shown in (C) \( rnl^{+} - rnl^{\Delta p} \). The solid line shows the segment 100 to 500 bp downstream of the promoter.
The results for both \textit{rrnI}D\textsubscript{23-5S} and \textit{rrnI}D\textsubscript{23-5S} supported the idea that the segment 100 to 500 bp downstream of the promoter had the greatest effect on the CT conversion rates throughout the \textit{rrnI} segment.

The CT conversion of rDNA in the $\Delta$smc mutant cells

We conjectured that the interaction of Smc with rDNA affected the structure of the transcribing rDNA segment, and it facilitated the formation of the ssDNA. To determine whether the loaded Smc affects

![Figure 4. The CT conversion rates of the \textit{rrnI}\Delta SS segment](image-url)
the rate of the ssDNA at the 100-500 bp segment downstream of the promoter, we analyzed the CT conversion rates in the Dsmc mutant cells. Our results showed that the CT conversion rates were drastically increased at the 100-500 bp segment of rDNA in the smc deletion mutant cells (Figure S10). This suggested that the bound Smc proteins protect the cytosine residues from sodium bisulfite or suppress the formation of the ssDNA segments.

The CT conversion of inactive genes during vegetative growth

We evaluated the difference in the effect of the non-denaturing sodium bisulfite treatment between the wild-type and the mutant rml genes. Because the mutant rml cells did not exhibit the same growth as
the wild-type rrn strains, and the mutant rrn strains exhibited slow cell growth, we analyzed the CT conversion rates of another chromosomal segment as an internal standard to clarify the effect of cell growth on the CT conversion rates. The spsA and the spsB genes are inactive during vegetative growth and are activated by a sporulation-specific sigma factor, σ^K, in response to the deterioration of nutrients in a growing environment. Thus, the spsAB segment is suitable for analysis of the effect of the non-denaturing sodium bisulfite treatment on cell activities other than transcription.

We analyzed the CT conversion rates of the DNA segment, including the spsAB genes in the wild-type rrn strain. Figures 6A and 6B show the whole profile of the CT conversion of the spsAB segment by both the single nucleotides and the sliding window analysis. The top and bottom strand nucleotides showed markedly higher CT conversion rates (more than 10%). In particular, the CT conversion rates were high in the region upstream of the promoter on the top strand. The average CT conversion rates of the spsAB segment were 4.20% on the top strand and 3.65% on the bottom strand (Table S7). These rates were less than those for the rml and rmlΔp genes. Thus, the CT conversion of a chromosomal segment decreased due to the inactivation of transcription.

Next, we analyzed the CT conversion rates of the rDNA strain. We obtained whole profiles of the CT conversion of the rDNA segment by sliding window analysis (Figure S8). The results showed that the rDNA segment profiles in the mutated rDNA strains were similar. Moreover, the profiles were also very similar to that in the 2 rrn strain harboring the wild-type rrn. In fact, we did not find any apparent differences when the CT conversion rates in the mutated rDNA strains were subtracted from those of the 2 rrn strain harboring the wild-type rrn (Figures 6C–6E and S9). The differences in the CT conversion rate between the wild-type and the mutated rrn strains were less than 0.7 percentage points. Thus, the deletion within the rml gene did not affect the CT conversion of the other genes on the genome.

Interaction of smc with a full-length of rDNA under an RNase H-overproduction condition

We applied the co-immunoprecipitation assay without any chemical reaction for cross-linking between a protein and DNA to analyze the interaction of the Smc-ScpAB complex with a full-length of rDNA (rrn) in a living cell. The assay was carried out using a cell in which two additional genes, rnhB and rnhC, could be expressed under an inducible promoter. rnhB, and rnhC are genes of B. subtilis that independently encode RNase H. RNase H catalyzes the cleavage of RNA in a hybrid of DNA and RNA—namely, the R-loop. If the R-loop is formed at the segment 100 to 500 bp downstream of the promoter of rDNA, increased production of RNase H will affect the formation of the ssDNA and result in a decrease in the interaction of the Smc-ScpAB with rDNA. We repeated the assay three times independently. When the gene expression of rnhB and rnhC was induced, the amount of recovered rDNA that participated with the Smc-ScpAB complex was clearly decreased in every experiment compared to non-inducible conditions, suggesting that overexpression of RNase H hindered the binding of the Smc-ScpAB complex on rDNA (Figure S11).

DISCUSSION

We used a combination of biochemical and genomic approaches to detect where chromosome DNA segments melt into ssDNA in a bacterial cell. Non-denaturing sodium bisulfite treatment effectively catalyzed the conversion of unpaired cytosines to thymines via uracils without any DNA denaturation treatment. The CT conversion induced by non-denaturing sodium bisulfite treatment showed high performance for the detection of ssDNA segments in the genome by using NGS. Our analyses demonstrated that bacterial genomic DNA melts into ssDNA in living cells in a largely unpredictable manner.

We applied the CT conversion rates of the spsAB segment as an internal standard. The differences in the CT conversion rate at the spsAB segment between the wild-type and the mutated rrn (0.67%) strains were less than those at the rrn segment (1.26%) (Table S7). Judging from the above, the reduction of the cell growth rate did not reflect the CT conversion rates. Next, therefore, we carried out the non-denaturing sodium bisulfite treatment until the progression of the cytosine deamination was nearly saturated and then performed next-generation sequencing, with an average of 11,000 reads, covering each cytosine on the PCR-amplified rDNA segment. As a result, we were able to obtain the actual CT conversion rate to overcome perturbation by the cell growth rate. Therefore, we conclude that the CT conversion rate differences at the rrn segment between the wild-type and the mutated
rrnI were directly affected by the efficiency of the ssDNA formation, which deteriorates due to the deletion mutations.

When we compared the CT conversion rates between the wild-type and the mutated rrnI gene, the difference in the rates led us to discover ssDNA segments in the bacterial rDNA under active transcription. Multiple ssDNA segments were formed throughout the rDNA segment under active transcription. Temporary hybridization between a new RNA transcript and the template DNA strand reduced the CT conversion rate.
The biased CT conversion rates of the ssDNA segments suggested RNA loop formation.

The ssDNA segment was essential for loading the Smc-ScpAB complex. Biases in the CT conversion rates between the top and the bottom strand were obvious in this segment. It is conceivable that the ssDNA segment was exposed by the R-loop formation in a manner dependent on the rRNA transcription. The results are consistent with previous reports that the bacterial SMC proteins bind to ssDNA and preferentially load onto the actively transcribing rDNA. It is likely that the Smc-ScpAB complex is loaded onto the ssDNA segment located 100 to 500 bp downstream of the rRNA promoter, although further biochemical experiments will be needed to confirm the idea. The segment seems to stably maintain its unwound state during active transcription. Smc suppressed the formation of the ssDNA, partly because the binding of Smc blocked the sodium bisulfite and partly because of the reannealing activity of Smc from ssDNA to dsDNA.

Malig et al. reported a sophisticated approach for identifying R-loops in the human genome by analyzing the CT conversion rates of consecutive cytosines. In addition, a nuclease- and bisulfite-based method has been applied to identify R-loops in a strand-specific manner in the mouse genome. In contrast, in the present study we were able to detect R-loops of the bacterial genome in a straightforward manner. It is conceivable that the R-loop formation during transcription directly reflects differences in the CT conversion rates between the coding DNA strand and the template DNA strand. If so, this could be because the bacterial genome structure is simpler than the human genome structure. Modified cytosine bases in bacterial genomes are restricted to a specific motif of DNA. DNA methyltransferase of B. subtilis 168 (BsuM) modifies cytosine in the sequence 5'-YTCGAR-3'. Nine cytosines in the sequence were located in the rRNA genes. In addition, bacterial chromatin does not form nucleosome or nucleosome-like structures. Thus, the properties of bacterial genomes make it easier to detect the R-loop formation during transcription by using the CT conversion rates. Our procedure used in this experiment could be applied for analysis of the genome dynamics of prokaryotes.

The deletions in the latter half of the rRNA gene affected the ssDNA formation at the segment 100 to 500 bp downstream of the rRNA promoter. Even the 51 bp deletion in the 5S encoding segment was effective in reducing ssDNA formation at the downstream segment of the rRNA promoter. The transcription activity increases by 3.5-fold in the rRNAΔ5S gene. The ssDNA formation depended on the transcription from the rRNA promoter. Therefore, our results suggested that the latter half of the rRNA gene could somehow affect the transcription at the region upstream of the rRNA gene. The transcribed rRNA, including 5S rRNA, forms the secondary structure of RNA. Moreover, the transcribed product develops a higher-order structure than the tertiary structure of transcribed products. It is a plausible explanation that the higher-order structure of the transcribed RNA, including 5S rRNA, would affect the R-loop formation at the ssDNA segment at 100 to 500 bp downstream of the rRNA promoter. Indeed, an R-loop was reported to be formed at the replication origin of the ColE1 plasmid and stabilized by the higher-order structure of a transcribed RNA.

We consider the ssDNA formation at the 100-500 bp segment downstream of the promoter to have promoted the interaction of the bacterial condensin to rDNA. Indeed, the 100-500 bp segment deletion mutations of rDNA hinder efficiencies of co-immunoprecipitation of the derivatives of pRRN, which are plasmids harboring the deleted rRNA segment, with the Smc-ScpAB complex, except for pRRN/D16-23. The amount of retrieved DNA of that plasmid is relatively higher than those of other deletion plasmids, even though the 100-500 bp segment is lost. The reason why pRRN/D16-23 is partially active in interaction with the Smc-ScpAB complex is because there are relatively higher CT conversion rates in the remaining segment. The CT conversion rates at the remaining 23S segment of rRNA/D16-23 were higher than that of the wild type. Such segments are shown as the minus CT conversion rates of the upper strand (Figure S5D). The D16-23 deletion mutation of rRNA could alter DNA structure or R-loop formation, thereby partially recovering the loss of the 100-500 bp segment. In any case, an effective ssDNA formation would be crucial for the interaction of rDNA with bacterial condensin.

Our results cannot distinguish whether the marked difference of the ssDNA formation at the 100-500 bp downstream of the promoter resulted from the direct binding of the Smc-ScpAB complex onto the
100-500 bp segment or binding of the Smc-ScpAB complex somewhere on rDNA. It is possible that the Smc-ScpAB complex binds to sites other than the 100-500 bp segment, and the Smc binding causes an alteration of DNA structure at the 100-500 bp segment. Further experiments with the combined investigations of biochemical analyses and Bisulfite sequencing will reveal more precise mechanisms.

High rates of CT conversion were detected in the *B. subtilis* genome, irrespective of transcription activity. The CT conversion rates by the single-nucleotide analysis fluctuated dramatically throughout the rDNA. The positions of the cytosines with the higher CT conversion rates were shared in common among the rDNA segments we analyzed. However, we could not find a prominent consensus motif in the flanking sequences of the cytosines with the higher CT conversion rates. In general, the AT-rich motifs tend to be locally melted with a complete disruption of base pairing. This was not the case in our present analysis. Instead, the flanking sequences of the cytosines with the higher CT conversion rates contained CC, CCC, CCCC, CCCCC, and CCCCCC motifs. The cytosines with the higher CT conversion rates were also detected in the *spsB* gene. Thus, melting of the rDNA was not necessarily dependent on AT content, even though the higher CT conversion was precisely reflected by the melting of dsDNA. In addition, transcription did not directly influence the higher CT conversion rates, based on similar results for the *rrnDp* mutant. It may be that DNA replication was involved in the ssDNA formation of the cytosine-rich motifs. Alternatively, these ssDNA segments may have been formed due to DNA breathing by torsion of a specific conformation rather than by thermal fluctuations. Genome-wide analysis of the CT conversion rate may clarify the correlation between a DNA sequence and a tendency to form ssDNA segments.

Members of the SMC protein family possess ssDNA-binding ability in addition to dsDNA-binding ability. For example, yeast cohesin can topologically load onto the ssDNA and thereby capture the secondary DNA to establish DNA-DNA interaction by the cohesin ring. Bacterial RecN, which is closely related to the SMC family of proteins, topologically loads onto both ssDNA and dsDNA, and has a preference for ssDNA. Yeast and human condensin recognize unwound DNA in highly transcribed genes, or ssDNA. Indeed, the ssDNA binding ability is an important property for the loading of SMC proteins. It is not yet clear how the ssDNA-binding ability of the SMC proteins contributes to the maintenance of chromosomal DNA. Bacterial SMC proteins utilize the ssDNA-binding ability for topological loading to specific sites on the genome DNA, such as the stable ssDNA segment. A thorough analysis of the ssDNA formation in the genome would help elucidate the function of the ssDNA binding of the SMC proteins for topological loading.

**Limitations of the study**

In this study, we demonstrate that the ssDNA segment is formed at the 100-500 bp downstream of the rDNA promoter due to active transcription, and the ssDNA segment is crucial for the Smc-ScpAB loading onto rDNA. It seems that the Smc-ScpAB complex topologically loads onto the exposed ssDNA segment in the R-loop. However, it remains an open question whether Smc-ScpAB directly loads on the ssDNA segment or not. In addition, there is no direct evidence for the formation of the R-loop at the segment. Further experiments are needed to clarify the loading site of Smc-ScpAB and the R-loop formation at rDNA.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Bacterial strains
  - Plasmid construction
- **METHOD DETAILS**
  - Cultivation of cells for sodium bisulfite treatment
  - Sodium bisulfite treatment
  - PCR amplification of the rDNA and the *sps* segment
REFERENCES

1. Dame, R.T., Rashid, F.-Z.M., and Grainger, D.C. (2020). Chromosome organization in bacteria: mechanistic insights into genome structure and function. Nat. Rev. Genet. 21, 227–242. https://doi.org/10.1038/s41576-019-0185-4.

2. Hirano, T. (2016). Condensin-based chromosome organization from bacteria to vertebrates. Cell 164, 847–857. https://doi.org/10.1016/j.cell.2016.01.033.

3. Nasmyth, K., and Haering, C.H. (2005). The structure and function of SMC and kleisin complexes. Annu. Rev. Biochem. 74, 595–648. https://doi.org/10.1146/annurev.biochem.74.082803.133219.

4. Uhlmann, F. (2016). SMC complexes: from bacteria: mechanistic insights into genome structure and function. Nat. Rev. Genet. 17, 399–412. https://doi.org/10.1038/nrg3906.

5. Yatskevich, S., Rhodes, J., and Nasmyth, K. (2019). Organization of chromosomal DNA by SMC complexes. Annu. Rev. Genet. 53, 445–482. https://doi.org/10.1146/annurev-genet-112618-043633.

6. Hirano, M., and Hirano, T. (1998). ATP-dependent aggregation of single-stranded DNA by a bacterial SMC homodimer. EMBO J. 17, 7139–7146. https://doi.org/10.1093/emboj/17.23.7139.

7. Hirano, M., and Hirano, T. (2008). Opening closed arms: long-distance activation of SMC ATPase by hinge-DNA interactions. Mol. Cell 21, 175–186. https://doi.org/10.1016/j.molcel.2005.11.026.

8. Keyamura, K., and Hishida, T. (2019). Topological DNA-binding of structural maintenance of chromosomes-like RecN promotes DNA double-strand break repair in Escherichia coli. Commun. Biol. 2, 413. https://doi.org/10.1038/s42003-019-0655-4.

9. Murayama, Y., Samora, C.P., Kurokawa, Y., Iwaki, H., and Uhlmann, F. (2018). Establishment of DNA-DNA interactions by the cohesin ring. Cell 172, 465–477.e15. https://doi.org/10.1016/j.cell.2017.12.021.

10. Niki, H., and Yano, K. (2016). In vitro topological loading of bacterial condensin MukB on DNA, preferentially single-stranded DNA rather than double-stranded DNA. Sci. Rep. 6, 29469–29511. https://doi.org/10.1038/srep29469.

11. Sutani, T., and Yanagida, M. (1997). DNA renaturation activity of the SMC complex implicated in chromosome condensation. Nature 388, 798–801. https://doi.org/10.1038/42062.

12. Sutani, T., Sakata, T., Nakato, R., Masuda, K., Ishibashi, M., Yamashita, D., Suzuki, Y., Hirano, T., Bando, M., and Shirahige, K. (2015). Condensin targets and reduces unwound DNA structures associated with transcription in mitotic chromosome condensation. Nat. Commun. 6, 7815. https://doi.org/10.1038/ncomms8815.

13. Moriya, S., Tsujikawa, E., Hassan, A.K., Asai, K., Kodama, T., Ogasawara, N., and Ogasawara, N. (1998). A Bacillus subtilis gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. Mol. Microbiol. 29, 179–187. https://doi.org/10.1046/j.1365-2958.1998.00919.x.

14. Soppa, J., Kobayashi, K., Noirrot-Gros, M.-F., Oesterhelt, D., Ehrlich, S.D., Dervyn, E., Ogasawara, N., and Moriya, S. (2002). Discovery of two novel families of proteins that are proposed to interact with prokaryotic SMC proteins, and characterization of the Bacillus subtilis family members ScpA and ScpB. Mol. Microbiol. 45, 59–71. https://doi.org/10.1046/j.1365-2958.2002.03012.x.

15. Gruber, S., and Errington, J. (2009). Recruitment of condensin to replication origin regions by ParB/Spo0J promotes chromosome segregation in B. subtilis. Cell 137, 685–696. https://doi.org/10.1016/j.cell.2009.02.035.

16. Sullivan, N.L., Marquis, K.A., and Rudner, D.Z. (2009). Recruitment of SMC by ParB parS organizes the origin region and promotes efficient chromosome segregation. Cell 137, 697–707. https://doi.org/10.1016/j.cell.2009.04.044.
21. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359. https://doi.org/10.1038/nmeth.1923.

25. Shah, S.S., Hartono, S.R., Chedin, F., and Heyer, W.-D. (2020). Bisulfite treatment and single-molecule real-time sequencing reveals 3-D-loop length, position and distribution. Elife 9, e59111. https://doi.org/10.7554/eLife.59111.

26. Yu, K., Chedin, F., Hsieh, C.-L., Wilson, T.E., and Lieber, M.R. (2003). R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nat. Immunol. 4, 442–451. https://doi.org/10.1038/nature02919.

27. Hayatsu, H., Wataya, Y., and Kazushige, K. (1970). The addition of sodium bisulfite to uracil and to cytosine. J. Am. Chem. Soc. 92, 724–726. https://doi.org/10.1021/ja00706a062.

28. Shapiro, R., Servis, R.E., and Welcher, M. (1970). Reactions of uracil and cytosine derivatives with sodium bisulfite. J. Am. Chem. Soc. 92, 422–424. https://doi.org/10.1021/ja00756a026.

29. Leela, J.K., Syeda, A.H., Anupama, K., and Gowrishankar, J. (2013). Rho-dependent transcription termination is essential to prevent excessive genome-wide R-loops in Escherichia coli. Proc. Natl. Acad. Sci. USA 110, 258–263. https://doi.org/10.1073/pnas.1213231110.

30. Yano, K., Wada, T., Suzuki, S., Tagami, K., Matsumoto, T., Shiwa, Y., Ishige, T., Kawaguchi, Y., Masuda, K., Akanuma, G., et al. (2013). Multiple rRNA operons are essential for efficient cell growth and sporulation as well as outgrowth in Bacillus subtilis. Microbiology 159, 2225–2236. https://doi.org/10.1099/mic.0.067025-0.

31. Eichenberger, P., Fujita, M., Jensen, S.T., Conlon, E.M., Rudner, D.Z., Wang, S.T., Ferguson, C., Haga, K., Sato, L.J.S., and Losick, R. (2004). The program of gene transcription for a single differentiating cell type during sporulation in Bacillus subtilis. PLoS Biol. 2, e328. https://doi.org/10.1371/journal.pbio.0020328.

32. Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidonenko, E., Marchadier, E., Hoebeke, M., Aymierich, S., et al. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in Bacillus subtilis. Science 335, 1103–1106. https://doi.org/10.1126/science.1206648.

33. Itaya, M., Omori, A., Kanaya, S., Crouch, R.J., Tanaka, T., and Kondo, K. (1999). Isolation of RNase H gene that are essential for growth of Bacillus subtilis 168. J. Bacteriol. 181, 2118–2123. https://doi.org/10.1128/JB.181.7.2118-2123.1999.

34. Malig, M., Hartono, S.R., Giafaglione, J.M., Sanz, L.A., and Chedin, F. (2020). Ultra-deep coverage single-molecule R-loop footprinting reveals principles of R-loop formation. J. Mol. Biol. 432, 2271–2288. https://doi.org/10.1016/j.jmb.2020.02.014.

35. Muller, B., and Sarma, K. (2021). A nucleosome- and bisulfite-based strategy captures strand-specific R-loops genome-wide. Elife 10, e65146. https://doi.org/10.7554/eLife.65146.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Penta-His Antibody, BSA free | Qiagen | Cat#34660; RRID: AB_2619735 |
| ECL anti-mouse IgG, HRP-linked antibody | GE Healthcare | Cat#NA931; RRID: AB_772210 |
| **Bacterial strains and virus strains** |     |           |
| Bacillus subtilis | Laboratory strain | 168 |
| **Chemicals, peptides, and recombinant proteins** | | |
| RNA later Solution | Thermo Fisher Scientific | AM7020 |
| TaKaRa EpiTaq HS (for bisulfite-treated DNA) | TaKaRa | R110 |
| TB Green Premix Ex Taq II (Tli RNaseH Plus) | TaKaRa | RR820 |
| Agencourt AMPure XP | Beckman Coulter | A63881 |
| CelLytic B Cell Lysis Reagent | Sigma-Aldrich | B7435 |
| Dynabeads Protein G | Thermo Fisher Scientific | DB10004 |
| **Critical commercial assays** | | |
| EpiTect Fast Bisulfite Kits | Qiagen | 59824 |
| EpiTect Fast LyseAll Bisulfite Kit | Qiagen | 59864 |
| NEBNext Ultra DNA Library Prep Kit for Illumina | New England Biolabs | E7370 |
| Qubit dsDNA BR Assay Kit | Thermo Fisher Scientific | Q32853 |
| MiSeq Reagent Kits v2 (500 cycles) | Illumina | MS-102-2003 |
| Bio-Rad Bradford Assay | Bio-Rad | 5000006 |
| **Deposited data** | | |
| Sequence data from next-generation sequencing | This study | Sequence Read Archive (DRA, https://www.ddbj.nig.ac.jp/dra/index-e.html) database under accession numbers (DRA010569 and DRA013785) |

**Experimental models: Organisms/strains**

- B. subtilis YAN13122; trpC2 ΔrrnHG1 Δrrm O 1 ΔrmD1 ΔrmET ΔrrmB2 ΔrrmW2 ΔrmJ1::cat ΔICEBs1 rrl" rrnG::spc
  - Yano and Niki, 2017 | MBS514 available at NBRP B. subtilis |
- B. subtilis YAN12675; trpC2 ΔrrnHG1 Δrrm O 1 ΔrmD1 ΔrmET ΔrrmB2 ΔrrmW2 ΔrmJ1::cat ΔICEBs1 rmlΔp rrnG::spc
  - Yano and Niki, 2017 | MBS487 available at NBRP B. subtilis |
- B. subtilis YAN12698; trpC2 ΔrrnHG1 Δrrm O 1 ΔrmD1 ΔrmET ΔrrmB2 ΔrrmW2 ΔrmJ1::cat ΔICEBs1 rmlΔSS rrnG::spc
  - Yano and Niki, 2017 | MBS486 available at NBRP B. subtilis |
- B. subtilis YAN12685; trpC2 ΔrrnHG1 Δrrm O 1 ΔrmD1 ΔrmET ΔrrmB2 ΔrrmW2 ΔrmJ1::cat ΔICEBs1 rmlΔ23′-SS rrnG::spc
  - Yano and Niki, 2017 | MBS485 available at NBRP B. subtilis |
- B. subtilis YAN12684; trpC2 ΔrrnHG1 Δrrm O 1 ΔrmD1 ΔrmET ΔrrmB2 ΔrrmW2 ΔrmJ1::cat ΔICEBs1 rmlΔ23-SS rrnG::spc
  - Yano and Niki, 2017 | MBS484 available at NBRP B. subtilis |
- B. subtilis YAN12668; trpC2 ΔrrnHG1 Δrrm O 1 ΔrmD1 ΔrmET ΔrrmB2 ΔrrmW2 ΔrmJ1::cat ΔICEBs1 rmlΔ16-23-SS rrnG::spc
  - Yano and Niki, 2017 | MBS483 available at NBRP B. subtilis |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Requests for further information will be fulfilled by the lead contact, Hironori Niki (hniki@nig.ac.jp).

**Materials availability**
*Bacillus subtilis* strains and plasmids used in this study are available from the National BioResource Project, *Bacillus subtilis*, JAPAN (http://www.shigen.nig.ac.jp/bsub/).

**Data and code availability**
Datasets from next-generation sequencing are available at the DNA Data Bank of Japan, DDBJ (Accession No.: DRA010569 and DRA013785). The computational script constructed in this study is available at GitHub (https://github.com/KoichiYanoNIG/pid3). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains

All the strains used in this study are listed in the key resources table. The 2rrn mutant, YAN13122, carries two copies of the rRNA gene (rrnA and rrnl). Various lengths of deletion in rDNA were introduced in the rrnl gene, as shown in Figure S2. A gene replacement technique was applied to substitute a gene of interest. The smc gene was replaced with the erm gene according to the technique. A linear DNA fragment for the gene replacement technique was constructed by using the Splicing by Overlap Extension PCR (SOE PCR). To overproduce RNaseH, we built the Pspac-rnhB-rnhC operon in which both the rnhB and the rnhC genes encoding RNaseH were placed under the control of the IPTG (isopropyl β-D-thiogalactopyranoside)-regulated promoter, Pspac. We constructed a linear DNA fragment containing tandemly arranged genes of rnhB and rnhC by using SOE PCR. The linear DNA fragment was used to transform YAN16141, in which the Pspac promoter was located downstream of the aprEerm gene, resulting in YAN17462. To test the expression of RnhB and RnhC, the gfp gene without a promoter was inserted downstream of the rnhB and rnhC genes, and the intensity of GFP was measured. An image of fluorescence microscopy showed that the intensity of GFP indeed increased in the presence of IPTG.

Plasmid construction

Plasmid DNA was constructed by using the in vivo ligation system, iVEC (in vivo E. coli cloning). We had used the pGETS118-t0-Pr-SfiI-pBR322 plasmids in which the replication initiator Rep is regulated by gene expression under the control of the IPTG-inducible promoter, Pspac. We substituted the Pspac promoter with the PspAs promoter to avert the IPTG induction of the Rep protein, resulting in pVEC2. The rrnl segment of pRRN17 was cloned into pVEC2, resulting in pRRN2.

METHOD DETAILS

Cultivation of cells for sodium bisulfite treatment

The B. subtilis cells were cultured in L medium. When the smc deletion mutant was cultivated, the SMG medium was used for a growth medium with modification. Cells were cultured overnight at 37°C with shaking, and the cells were diluted to be an optical density 600 (OD600) of 0.04. When OD600 reached 0.4, one mL of the culture was used for the sodium bisulfite treatment. After the centrifugation, the supernatant was discarded. The cell pellet was resuspended in 500 μL of an RNA stabilizing reagent (RNA later; Thermo Fisher Scientific) and incubated at room temperature for 10 min. After centrifugation and discarding the supernatant, the cell pellet was frozen in liquid nitrogen and stored at −80°C until use.

Sodium bisulfite treatment

Aqueous 80% methanol (methanol: water, 80:20 v/v) was directly added to the frozen cell pellet and left for 5 min, and then the cells were collected by centrifugation. After discarding the supernatant, the cells were lysed with lysozyme at concentrations of 0.4 μg/mL in a buffer (10 mM Tris-HCl, pH 8.0, 4 mM EDTA, 4% (w/v) sucrose) for 30 min at 37°C. Sodium bisulfite treatment was carried out using commercially available bisulfite conversion kits (EpiTect Fast Bisulfite Kit and EpiTect Fast LyseAll Bisulfite Kit; Qiagen) according to the manufacturer’s instructions except for the DNA denaturation step and sodium bisulfite reaction time. We carried out the sodium bisulfite reaction at 45°C without DNA denaturation, and incubated it for 120 min. The incubation time was determined according to the CT conversion rates after a series of the sodium bisulfite reactions with incubations ranging from 0 to 180 min (Figure S12). After the bisulfite-treated DNA was eluted from the DNA spin column, the DNA was precipitated with ethanol, rinsed with 70% ethanol, resuspended with Milli-Q water, and frozen at −80°C until use. Reagents were prepared just before using.

PCR amplification of the rDNA and the sps segment

To determine CT conversion rates in the whole region of the rrnl gene, the region divided into 5′- and 3′-half were separately amplified by PCR using the primers listed in Table S8. To amplify the 5′-half of the rrnl gene, specific primers that hybridize to the region upstream of the rrnl promoter (#1) and the inside of the gene encoding 23S rRNA (#2) were used. To amplify the 3′-half of the rrnl gene, specific primers that hybridize to the inside of the gene encoding 23S rRNA (#3) and the spectinomycin-resistance gene (spc), which was inserted at the region downstream of the rrnl gene (#4), were used. To determine CT conversion rates at the spsAB genes, specific primers that hybridize to the upstream region of the spsA promoter (#5) and the inside of the spsB gene (#6) were used. The amplified segments are shown in Figure S13.
Library preparation and sequencing with MiSeq

To amplify the regions for sequencing, the target DNA fragments were amplified by PCR (TaKaRa EpiTaq HS; TAKARA). The PCR fragment was purified by precipitation with PEG 8000. The concentration of the DNA fragments was quantified by using a fluorescence dye and a fluorometer (Qubit dsDNA BR assay kit; Thermo Fisher Scientific). The amplified DNA fragments (100 ng) were sheared to the average length of 250 bp by using an ultrasonicator (Covaris focused-ultrasonicator S220; Covaris) and purified by using magnetic beads (Agencourt AMPure XP; Beckman Coulter). Adaptor and index primers were added to the sheared DNA fragments using a next-generation sequencing library preparation kit (NEBNext Ultra DNA Library Prep Kit for Illumina; NEB) according to the manufacturer’s instructions. PCR enrichment was carried out with nine cycles. The index-ligated fragments were mixed to construct a sequencing library and sequenced using the MiSeq system and its reagent kit (MiSeq Reagent Kits v2 (500 cycles); Illumina) according to the manufacturer’s instructions. Sequencing was carried out with 250 cycles and a paired-end mode.

Determination of the parameters for mapping of the sequenced reads

Sequenced reads were first trimmed using a trimming tool, Trimmomatic (v0.33). To trim the reads, the following parameters were used: accepted mismatches, 2; palindrome clip threshold, 30; simple clip threshold, 10; leading, 20; trailing, 20; window size, 4; average quality, 15; minimal length, 30. The trimmed reads were mapped to the sequence of the 5’ half of rrnI as a reference by using mapping software, Bismark (v0.20.0). The version of Bowtie2, the software required to run Bismark, was v2.2.6. In order to map as many reads as possible, we determined parameters for the minimum alignment function of Bismark (score-min option). The option consists of three parameters: (a) a function type, (b) a constant term, X, and (c) a coefficient, Y. We used a linear equation (L) for the function type and ran Bismark with score-min L, -X, and -Y, where X and Y were changed from 0 to 150 and 0 to 4, respectively. While Y was being changed, X was fixed as 0. While X was being changed, Y was fixed as 2. The mapping efficiencies were plotted against the increasing values of X or Y (Figure S14). The values of X and Y required to map as many reads as possible were determined as 100 and 2, respectively.

Quality control of the mapped reads

Mapped reads below MAPQ<= one were removed using SAMtools (v1.9), and reads with insertions and deletions were removed using an in-house Perl script pid3.pl.

Calculation of the CT conversion rates

Depths of the coverage for each nucleotide position in the mapped reads were counted for four bases (A, T, G, C) by using SAMtools. CT conversion rates were calculated for each cytosine on both the top and bottom strands according to the following equations: CT conversion rates for top strand (%) = (Counts for T)/(Counts for C + Counts for T) × 100; CT conversion rates for bottom strand (%) = (Counts for A)/(Counts for G + Counts for A) × 100.

Pull-down assay for Smc-ScpAB binding to DNA

Cells were cultivated overnight in L medium containing tetracycline (10 μg/mL) with shaking at 37°C. The overnight culture was diluted to 100 mL of fresh L medium containing tetracycline until OD600 of 0.005. In half an hour, IPTG was added to the culture (a final concentration of 0.5 mM). When OD600 reached 0.6, the culture was diluted to 600 mL of fresh L medium containing tetracycline until OD600 of 0.05. When the OD600 reached to 0.6, the culture was centrifuged. The supernatant was removed, and the cell pellet was frozen with liquid nitrogen and stored at −80°C until use.

Pull-down assay was performed essentially as described previously and the details are described below; The frozen cell pellet was resuspended in 6 mL of buffer A: 50 mM HEPES- KOH (pH 7.6), 100 mM KCl, 1 mM PMSF, 0.33 mg/mL lysozyme, 3.3% (w/v) sucrose, 8.3 mM Tris-HCl (pH 7.5), 0.05 mg/mL RNaseA, and 2 mM MgCl2. After incubation at 37°C for 10 min, the cell suspension was frozen and thawed three times using liquid nitrogen. To lyse cells, 6 mL of a cell lysis reagent (CelLytic B, Sigma-Aldrich) was added, and the solution was gently mixed and then incubated on ice for 10 min. The cell lysate was centrifuged at 9,100 × g for 10 min at 4°C. The supernatant was mixed with 37.5 μL of magnetic beads coated by Protein G (Dynabeads, Thermo Fisher Scientific) to eliminate organic matter that nonspecifically binds to the magnetic beads. The Dynabeads magnetic beads had been previously equilibrated with buffer B (50 mM
HEPES-KOH [pH 7.6], 100 mM KCl) and resuspended at a concentration of 30 mg/mL. A mixture of cell lysate and beads was incubated at 4°C for 2 h using a rotator. The mixture was placed in a magnetic stand. The supernatant was transferred to a new tube. To isolate histidine-tagged ScpB, mouse anti-pentahistidine tag antibody (QIAGEN, Germany) and Dynabeads protein G (30 mg of Dynabeads per 1 mL) were mixed (100 mg of the antibody per 1 mL of Dynabeads) and incubated for 1–2 h at 4°C with stirring by a rotator. The mixture was washed with buffer B and resuspended in buffer B (final concentration of 30 mg of Dynabeads per 1 mL). The antibody-coated Dynabeads (37.5 μL) were added to the supernatant and incubated overnight at 4°C with stirring by a rotator. The tubes containing Dynabeads were placed in a magnetic stand, washed three times with 3 mL of buffer B, washed once with 3 mL of buffer C (50 mM HEPES-KOH [pH 7.6] and 500 mM KCl), resuspended in 1.5 mL of buffer B, and transferred to new microcentrifuge tubes. The tubes were placed in a magnetic stand, and the supernatant was discarded. To elute proteins bound to Dynabeads, 200 μL of elution buffer (50 mM HEPES-KOH [pH 7.6], 10 mM EDTA, and 1% SDS) was used.

The amount of the retrieved plasmid DNA was measured by a real-time experiment (qPCR) using a thermal cycler Dice real-time system (model TP800; TaKaRa) and TB Green (TaKaRa) as a reporter dye. We made a series of diluted solutions of plasmid DNAs which were purified from E. coli cells and whose concentrations were quantified by using Qubit Fluorometer (ThermoFisher Scientific), then used for a standard curve of qPCR. We drew respective standard curves for each plasmid i.e. pVEC2 and pRRN2.

The amount of histidine-tagged ScpB protein in eluates was quantified with Western blotting. Purified histidine-tagged ScpB protein was used to derive a standard curve for absolute quantification. The concentrations of purified histidine-tagged ScpB were determined by Bio-Rad Bradford Assay (Bio-Rad) using BSA as a standard. For detection of Western blotting, mouse anti-pentahistidine tag antibody (Qiagen) and ECL anti-mouse IgG, HRP-linked antibody (from sheep; GE Healthcare) were used as a primary and secondary antibody, respectively. The intensity of chemiluminescence on Western blotting membranes was detected using a Chemiluminescence CCD Imaging System (Luminograph WSE-6110H; Atto Corp.) and the band intensity was quantified with ImageJ software.

We determined the ratio of antibiotic-resistant cells in liquid culture as an index of plasmid stability. An aliquot of liquid culture was spread onto L agar plates and incubated at 37°C. Each single colony (at least 300 colonies) was streaked on L agar plates with or without tetracycline (15 μg/mL).

The quantified amounts of the retrieved plasmid DNAs were corrected by using the amount of histidine-tagged ScpB protein and plasmid stability.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The mean value and standard deviations of the mean were calculated. Simple regression analysis was done to draw regression lines in the correlation plots between cytosine contents and CT conversion rates. The equations of the regression line for the top and bottom strand are y = 22.405x + 1.9114 and y = 18.677x + 0.8789, respectively, where x is the cytosine content and y is the CT conversion rate.