Two DNA Translocases Synergistically Affect Chromosome Dimer Resolution in Bacillus subtilis

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In Bacillus subtilis, chromosome dimers that block complete segregation of sister chromosomes arise in about 15% of exponentially growing cells. Two dedicated recombinases, RipX and CodV, catalyze the resolution of dimers by site-specific recombination at the dif site, which is located close to the terminus region on the chromosome. We show that the two DNA translocases in B. subtilis, SftA and SpoIIIE, synergistically affect dimer resolution, presumably by positioning the dif sites in close proximity, before or after completion of cell division, respectively. Furthermore, we observed that both recombinases, RipX and CodV, assemble on the chromosome at the dif site throughout the cell cycle. The preassembly of recombinases probably ensures that dimer resolution can occur rapidly within a short time window around cell division.

During the course of the bacterial cell cycle, chromosomes need to be faithfully replicated and distributed to ensure that both daughter cells inherit a complete copy of the genetic information. However, the replication of circular chromosomes can lead to a covalently interlinked form of the chromosome (dimer), which results from an odd number of recombination events during replication and cannot be segregated. In Escherichia coli, dimers are resolved by an elaborate system of site-specific recombinases (XerD and XerC), for recombination at the dif site, which is located near the terminus region of the chromosome (22). XerC and XerD are both members of the tyrosine recombinase family and catalyze the formation and resolution of a Holliday junction intermediate at the dif site where each recombinase mediates a strand exchange reaction (7). The action of the XerCD recombinases is regulated and facilitated by the FtsK protein, which arranges the dif sites in close proximity and directly activates XerD. FtsK is recruited to the division septum as a component of the cell division machinery via its N-terminal domain, which anchors the protein in the membrane and is additionally essential for cell division (9, 30). The C-terminal domain is an ATP-dependent DNA translocase that moves DNA at very high rates (15, 19) and in a spatially directed manner. Directionality of DNA translocation is predetermined by the orientation of short polar sequences on the chromosome (FtsK-orienting polar sequences [KOPS]), which are recognized by the FtsK domain and permit the loading of FtsK onto the DNA in one specific orientation (14). KOPS are distributed over the chromosome and oriented toward the terminus of replication, where they are found at a high frequency (3). By KOPS-guided directed DNA translocation, FtsK arranges the duplicated dif sites in close proximity at the division septum and thereby facilitates dimer resolution (4). Additionally, FtsK directly activates the catalytic state of XerD and is therefore essential for effective chromosome dimer resolution (1, 28).

The concept of site-specific recombination to resolve chromosome dimers is widely conserved among bacteria and archaea, and in most cases bacteria with homologues to XerD and XerC contain also an FtsK homologue. In Bacillus subtilis, the homologues of XerD and XerC are RipX and CodV, respectively, which share 35% and 44% sequence identity with their E. coli counterparts (18, 21). Both RipX and CodV were reported to bind to the B. subtilis dif site and to catalyze strand exchange in vitro, which suggests a role similar to that of E. coli XerD and XerC (20), but a possible function for a DNA translocase similar to FtsK during dimer resolution in B. subtilis has not been clarified so far. In B. subtilis two DNA translocases are involved in the rescue of DNA that might become trapped by the division septum during vegetative growth: the membrane-associated SpoIIIE protein and the soluble SftA protein (11). The two proteins share substantial sequence identity with the C-terminal domain of FtsK and hence could participate in dimer resolution. However, in vivo studies revealed that neither SpoIIIE nor SftA (formerly named YtpT) is essential for RipX-dependent recombination at dif (5, 13, 20). In this study, we wanted to gain further information on the role of the DNA translocases SftA and SpoIIIE in B. subtilis dimer resolution. We observed that SftA and SpoIIIE both have an impact on the process of dimer resolution, acting in synergy, most likely by positioning the dif sites in close proximity. Furthermore, our results indicate that the site-specific recombinases RipX and CodV form a preassembled complex on the chromosome during the cell cycle, probably to allow an immediate initiation of dimer resolution.

MATERIALS AND METHODS

Growth conditions and construction of strains. The bacterial strains and plasmids used in this study are listed in Table 1. Bacillus subtilis strains were derived from wild-type strain PY79 (29) or YB886 (27). Escherichia coli strain XL1-Blue (Strategene) was used for the construction and propagation of plasmids. Cells were grown in Luria-Bertani rich medium at 30°C or 37°C, supplemented with antibiotics where appropriate (ampicillin, 100 μg ml−1; chloramphenicol [Cm], 5 μg ml−1; spectinomycin [Spc], 100 μg ml−1; tetracycline [Tet],

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CK210 was transformed with chromosomal DNA of the strain SL8526 (from P. Piggot) to generate strain KS82. (Tetr) terminus, competent cells of strains CK209 and CK168 were transformed with chromosomal DNA of strain CK168 and selected for spectinomycin resistance. For colocalization of RipX-YFP with the terminus region of the chromosome, strain CK209 was combined with a lacO cassette at 181° (25) and a Lac-cFP variant expressed from a constitutive promoter (laboratory stock).

**Growth competition assay.** Growth competition assays were carried out as described previously (16, 26). Briefly, two strains to be tested were mixed at a 1:1 ratio and grown in 20 ml LB medium for about 60 to 80 generations at 30°C. Every 12 h (approximately 10 generations), cultures were diluted 1:1,000 in fresh medium and the number of CFU of each competitor was determined by plating serial dilutions on selective agar plates. To compare the fitnesses of mutant and parent strains, we calculated the coefficients k from the exponentials describing the ratio of the mutant strain versus its parent, R = Nm/Np, as a function of the number of generations, n, using the formula k = ln(R)/n (see Fig. 2). For small values (k < 0.2), k equals 1 − e−k, hence giving the frequency that a mutant strain fails to produce a viable cell compared to its parent at each generation (26).

**Plasmids**

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**RESULTS AND DISCUSSION**

Formation of chromosome dimers in ΔripX, ΔrecA, and ΔripX ΔrecA mutants. We used fluorescence microscopy to determine the phenotypic effects related to chromosome partitioning in ΔripX and ΔrecA mutants as well as ΔripX ΔrecA double mutants of *B. subtilis* strain PY79. Cells grown to exponential phase at 30°C in LB medium were treated with fluorescent live stains for DNA and membrane and were analyzed microscopically. The ΔripX mutant showed partitioning defects in 14.2% of cells (Table 2) (1,083 cells were analyzed). The defects were unsegregated nucleoids in large cells and chromosomes that were bisected by a division septum (Fig. 1A, middle left panel), as well as anucleate cells. The partitioning defects were significantly reduced to 7.5% in the ΔripX ΔrecA double mutant cells (P < 0.001; 703 cells were analyzed) (Fig. 1A, right panel). We also observed considerable partitioning defects in 4.9% of recA mutant cells (Fig. 1A, middle right panel, and Table 2), whereas in a previous study we detected only 0.4% of partitioning defects in the PY79 wild type under
the same conditions (11). These data show that the effects of \(\Delta \text{ripX}\) are largely relieved in the absence of RecA-dependent recombination in \(B. \text{subtilis}\) (Table 2), and it was inferred that RecA-dependent homologous recombination is the main route for dimer formation in \(B. \text{subtilis} \) PY79. However, we also detected unsegregated chromosomes that could account for unresolved chromosome dimers in the \(\Delta \text{ripX} \Delta \text{recA}\) double mutant. One possible explanation is the formation of dimers...

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independently of recA, for example, by a phage-encoded recombinase. Moreover, it is difficult to microscopically distinguish chromosome dimers and chromosome catenates, which are interlinked forms of two chromosomes that also prevent segregation and which were observed before in E. coli recA mutants (24, 31). We obtained similar results with a different B. subtilis wild-type strain, YB886, which is cured of the SP/H9252 phage (see Table S1 and Fig S1A in the supplemental material). The relative numbers of partitioning defects differed slightly between PY79 and YB886 (Table 2; see Table S1 in the supplemental material), indicating some variability among different B. subtilis strains.

Our observations are in agreement with an earlier study by Lemon et al. (13), which described a significant reduction of anucleate cells in a recA mutant compared to the single mutant of B. subtilis strain JH642. We suppose that the finding that partitioning defects of the recX mutant are exaggerated in the recX:recA mutant, which was reported in a study by Sciochetti et al. (21), is due to the strain background (for example, many phage-like elements contain additional Rec proteins) and that our findings are valid for many other B. subtilis strains.

**Determination of the relative fitnesses of mutant strains.** We compared the fitnesses of different mutant strains in coculture growth experiments. Mutant strains compete with the parent strain for several generations, and differences in fitness can be observed even when a relatively small subset of the population is affected (16, 23). A difference in fitness is represented by the growth coefficient $k$ and can be the result of an increased generation time of the mutant strains as well as the production of nonviable cells. Since the mutant strains used here differ only slightly in generation time (see Table S2 in the supplemental material), one can infer that $k$ reflects mainly the number of nonviable cells produced in each cell division. E. coli mutants with mutations in xerD or ftsK show reduced fitness when competing with the wild-type strain (2, 28). However, when recA is deleted additionally, double mutants have no disadvantage compared to the recA single mutant, because in the absence of RecA-dependent homologous recombination, no chromosome dimers are formed and dimer resolution is dispensable.

The recA mutant strain resulted in a growth coefficient of 0.22 when competing with wild type cells. The generation times between the strains differed only slightly, by 2 min, indicating that a recA mutant culture produces about 20% of nonviable cells at cell division. Indeed, we observed a considerable partitioning defect by microscopy (Table 2). The nonviable cells probably are the result of stalled replication forks, which cannot be repaired without RecA. A similar effect on plating efficiency and partitioning phenotypes observed by microscopy for a recA mutant was reported before by Sciochetti et al. (21).

**FIG. 2. Growth competition assay.** Different deletion mutants of B. subtilis PY79 were grown in coculture with their parent strain, either wild type (black circles) or ΔrecA (white circles), for several generations. (A) Upper panel, ΔripX mutant. Lower panel, competition of two ripX deletion strains, in which ripX is deleted by different antibiotic cassettes (Cm or Tet). (B) ΔsftA (upper panel), ΔspoIIIE (middle panel), and ΔsftA ΔspoIIIE (lower panel) DNA translocase mutants. The ratio of CFU (mutant/parent) was determined by plating on selective agar plates and is plotted against the number of generations. For details, see the text. The data represent at least two independent experiments.
results are in accordance with our observations from fluorescence microscopy (Fig. 1A), where we detected that partitioning defects in a ΔripX mutant strain were relieved in a ΔripX ΔrecA mutant (Table 2). Similar results were obtained for a growth competition assay using wild-type strain B. subtilis YB886 (see Fig. S1B in the supplemental material). As controls for these experiments, we used tetracycline (Tet) or chloramphenicol (Cm)-resistant ripX mutant strains and a strain expressing spectinomycin (Spc) resistance from the amyE locus. Cm and Tet cassettes showed an average difference (k) of 0.03 (Fig. 2A, lower panel), while Spc resistance did not influence the plating efficiencies of the B. subtilis strains used (data not shown), showing that strain-to-strain variations concerning resistance cassettes are considerably smaller than differences between deletion mutants and wild-type strains.

To address the role of the DNA translocases SftA and SpoIIIE in dimer resolution in B. subtilis, the fitnesses of ΔsftA and ΔspoIIIE mutant strains when competing with the parent wild-type or ΔrecA mutant strain were determined. Both the ΔsftA (k = 0.06) and ΔspoIIIE (k = 0.08) mutants showed reduced fitness compared to the wild-type strain (Fig. 2B). This corroborates observations made earlier by fluorescence microscopy where the ΔsftA and the ΔspoIIIE mutants strain showed a mild defect in chromosome segregation (11), which accounts for the reduced fitness now determined by growth competition.

In a ΔrecA mutant background, the reduced fitnesses caused by ΔsftA and ΔspoIIIE was largely restored (Fig. 2B). The growth coefficients (k) observed were 0.02 for the ΔsftA ΔrecA strain and 0.03 for the ΔspoIIIE ΔrecA strain, which is within the range of differences that can be produced by different resistance cassettes. This indicates that the phenotypic effects due to loss of DNA translocases are reduced in the absence of RecA-dependent homologous recombination and chromosome dimer formation. The action of each DNA translocase therefore affects the process of dimer resolution in B. subtilis. However, the effect caused by the deletion of a DNA translocase is not as severe as for ΔripX, confirming that SftA or SpoIIIE is not essential for dimer resolution, which has been reported before (5, 20).

Importantly, the deletion of both DNA translocases led to a growth coefficient of 0.15 and therefore had an additive effect over the single mutations. These data reveal that the presence of at least one DNA translocase, SftA or SpoIIIE, is essential for dimer resolution in B. subtilis PY79 and that SftA and SpoIIIE confer redundant functions. When recA was deleted additionally, the ΔsftA ΔspoIIIE ΔrecA triple mutant still showed a growth disadvantage (k = 0.06) versus the parent ΔrecA (Fig. 2B). This finding implies that although the disadvantage caused by loss of SftA and SpoIIIE is as severe as that in the ΔripX mutant, both DNA translocases also have a function in processes other than dimer resolution, because they are also important in the absence of RecA-dependent homologous recombination, where RipX is redundant. The fact that fitness is not fully restored in the triple mutant suggests that chromosomes are trapped by the division septum also for reasons other than dimer formation, and therefore, translocases are needed to efficiently complete chromosome segregation.

SftA and SpoIIIE are likely essential for dimer resolution by arranging the dif sites in close proximity by directed DNA translocase activity, similar to the case for FtsK. The amino acid sequence motif responsible for DNA recognition is highly conserved between FtsK, SpoIIIE, and SftA, and several short polar sequences similar to KOPS are found on the B. subtilis chromosome oriented from origin to terminus (17). For SpoIIIE, recognition of polar sequences and directed DNA translocation have been shown in a single-molecule experiment (17), and SftA possesses DNA-dependent ATPase activity (11). SftA and SpoIIIE are required to rescue bisected chromosomes that become trapped by the division septum due to a delay in segregation (5, 11), making them ideally suited for driving dimer resolution.

RipX and CodV are associated with the chromosome throughout the cell cycle. We monitored a fluorescently labeled variant of the site-specific recombinase RipX in vivo to gain information on its localization within the cell and its association with the chromosome. The strain that we constructed expresses RipX-YFP as the sole coprotein of the protein from its original promoter. The fluorescent variant of RipX retained about 80% of the activity of the native protein, as 2.7% of the cells (n = 863) showed a partitioning defect, in contrast to 14.2% of cells of a ΔripX deletion mutant and 0.4% of wild-type cells under the same conditions (Table 2 and data not shown). RipX-YFP was invariably associated with the nucleoid and formed a small, distinct focus (or two foci) on the chromosome in 65% of all cells (Fig. 1B). In cells which had completed replication and showed two separate chromosomes, two RipX-YFP foci were observed (Fig. 1B, inset). In a few cases (fewer than 2% of the cells), a single RipX-YFP focus was observed between two separated nucleoids, likely representing active dimer resolution taking place between interlinked chromosomes. Thus, dimer resolution appears to occur very rapidly within a short time window around cell division.

A localization pattern similar to that of RipX was observed for the second site-specific recombinase involved in chromosome dimer resolution, CodV. CodV-YFP also localized as a single distinct focus on the chromosome in small and medium-sized cells and as one or two foci in large cells (Fig. 1C). These findings suggest that both site-specific recombinases form a preassembled complex at a distinct site on the chromosome. However, the very faint YFP and CFP signals precluded testing the colocalization of the proteins.

Because not all cells showed RipX-YFP foci, we wished to determine if RipX-YFP is loaded onto the chromosome at a certain step in the cell cycle (e.g., after replication) or is associated with the chromosome throughout the cell cycle. RipX-YFP foci are very faint and can easily get lost when they are below the focal plane, so the one-third of the cells lacking foci may represent such cases. Cells were grown with a doubling time of 65 min to ensure single rounds of replication. To test this, we constructed a strain which allows the observation of RipX-YFP and the origin region (lacO1/LacI-CFP) simultaneously. If RipX was loaded onto the chromosome at a certain time point, we would expect to observe signals only in cells of certain sizes (and thus ages). From 120 cells analyzed, 82 showed clear RipX-YFP as well as LacI-CFP foci. We found significant numbers of examples of an association of RipX-YFP with the chromosome, which represent the whole cell cycle (Fig. 3). We observed small cells (16 of 82 cells analyzed) with a single origin-CFP and RipX-YFP focus, which indicates
that RipX-YFP is associated with the chromosome before replication has been initiated. Most of the cells (46 of 82) were undergoing replication and showed two origin-CFP foci and a single RipX-YFP focus. Furthermore, we observed cells with two origin-CFP foci and two RipX-YFP foci (20 of 82 cells), which have completed replication but have not yet initiated cell division (Fig. 3B). We measured the distance from origin-CFP and RipX-YFP foci to the cell pole to specify their position within the cell (Fig. 3A). Origins were segregated toward the cell poles during replication, while RipX remained at the midcell position (25). We measured the distance from origin-CFP and RipX-YFP foci to the cell pole to specify their position within the cell (Fig. 3A). Origins were segregated toward the cell poles during replication, while RipX remained at the midcell area or closer to the division site, which is consistent with the localization of the chromosomal terminus region (25).

In a previous report, the \textit{B. subtilis} dif site was identified to be located close to the terminus region at 166° (20). Furthermore, the authors demonstrated a specific interaction of RipX with the \textit{dif} site \textit{in vitro} using purified RipX protein. To test if RipX might bind to the \textit{dif} site \textit{in vivo} as well, we used the lacO/LacI-CFP reporter system to mark the terminus region (181°) of the chromosome in a strain concomitantly expressing RipX-YFP. In a majority of cells (344 of 350 cells analyzed), RipX-YFP localized in close proximity to the terminus, suggesting that RipX indeed binds to the \textit{dif} site in \textit{B. subtilis} cells (Fig. 1D). To support this notion, we moved the RipX-YFP fusion into a strain in which the \textit{dif} site had been deleted (SL8420) (20). As expected, we did not observe any RipX-YFP foci in the \textit{Δdif} mutant strain (Fig. 1E). Similar to the case for RipX, the foci formed by CodV-YFP were always found in close proximity to the terminus region in a wild-type background (see Fig. S2A in the supplemental material) but were absent in a \textit{Δdif} mutant strain (see Fig. S2B in the supplemental material).

**RipX-YFP rarely colocalizes with DNA translocases.** If SftA and SpoIIIE are to interact with RipX/CodV and thereby position the \textit{dif} site at the cell center, we would expect colocalization of the enzymes in a significant number of cells, as 15% of the cells generate dimers at the end of the cell cycle. Colocalization of SftA-CFP, which is a component of the cell division machinery, with RipX-YFP revealed that when division is initiated (as revealed by the midcell signal of SftA), RipX was located mostly in the separate cell halves, in accordance with the localization of the chromosome terminus region after successful segregation (Fig. 1F, cell I) (25). RipX-YFP localized to the cell middle, presumably when chromosome dimers were formed, but mostly adjacent to the division septum and to SftA (15 of 337 cells), and only in rare cases colocalized with SftA (3 of 337 cells). Figure 1F shows a cell (cell II) where SftA-CFP present at midcell is flanked by two adjacent RipX-YFP foci, potentially resuming chromosome segregation shortly after resolution of a dimer. These data suggest that dimer resolution occurs very rapidly, such that the process can hardly be observed in live cells. To test if SftA may colocalize with RipX more frequently when dimer resolution is blocked ("frozen"), we monitored the localizations of RipX-YFP and SftA-CFP in \textit{ΔcodV} mutant cells. In the absence of CodV, RipX-YFP still formed foci (Fig. 1G), showing that it assembles at the \textit{dif} site independently of its partner recombinase. However, some cells contained more than two RipX-YFP foci (Fig. 1G, lower panels), likely because chromosome dimers accumulate and block cell division. Interestingly, in the \textit{codV} deletion background, SftA-CFP and RipX-YFP colocalized in 10.5% of all cells having RipX foci (35 cases of colocalization among 335 cells). Thus, blocking of dimer resolution increases the frequency of colocalization of RipX and the DNA translocase. These experiments support the views that (i) resolution of chromosome dimers occurs very rapidly and (ii) SftA likely moves along the DNA around the terminus site in a KOPS-like mechanism, thus positioning it close to midcell. We favor the view that SftA and SpoIIIE do not specifically activate RipX/CodV, as is the case for FtsK in \textit{E. coli}, because in the absence of either translocase, dimer resolution can still occur (although less efficiently) and because even during a block in resolution, colocalization is not overwhelming. However, our experiments cannot resolve this point, and possibly both SftA and SpoIIIE are able to specifically activate RipX/CodV and resolution is simply too fast to be captured microscopically.

**FIG. 3.** Localization of RipX-YFP during the cell cycle. (A) Distance from the origin region and RipX to the last division site, dependent on the cell length. White triangles, origin-CFP focus; black circles, RipX-YFP focus. The dashed line represents the midcell position, and the continuous line represents the cell pole. Exemplary data corresponding to cells I, II, and III in panel B are highlighted in color. Forty-one cells were analyzed. (B) Simultaneous localization of RipX-YFP (green) and a lacO/LacI-CFP reporter system marking the origin region (359°) of the chromosome ("origin-CFP") (red). I, cell with one origin-CFP focus and one RipX-YFP focus; II, cell with two origin-CFP foci and one RipX-YFP focus; III, cell with two origin-CFP foci and two RipX-YFP foci. Bars, 2 μm.
SpolIIIE, the second DNA translocase in *B. subtilis*, functions in the rescue of septum-entrapped DNA when cell division is completed. A variant of SpolIIIE fused to mCherry forms a bright focus at the septum when DNA is trapped (11) and is otherwise distributed over the cell membrane. RipX-YFP foci could be observed close to the septum and close to a SpolIIIE-mCherry focus when DNA was visibly bisected by the septum (Fig. 1H, upper panels). However, in most cases (34 of 40 cells with a SpolIIIE focus), RipX-YFP and SpolIIIE-mCherry foci were well separated, either because dimers had been fully resolved earlier or because DNA was trapped by the division septum during the normal course of chromosome segregation (Fig. 1H, lower panels).

Conclusions. In toto, we show that the two DNA translocases in *B. subtilis*, SftA and SpolIIIE, are vital for the resolution of chromosome dimers, conferring additive functions in the process. From phenotypic analysis it was proposed earlier that individually, SpolIIIE and SftA facilitate dimer resolution (5, 13). We show that both DNA translocases synergistically enhance dimer resolution, revealing that in contrast to *E. coli*, *B. subtilis* employs two DNA translocases for dimer resolution, and the absence of both enzymes strongly compromises the process. Interestingly, we observed that the RipX and CavD recombinases are assembled on the chromosome close to the terminus, presumably at the dif site, throughout the cell cycle. The preassembly of the catalysts suggests that dimer resolution is set up to occur as rapidly as possible. Our cytological data suggest that the DNA translocase SftA facilitates dimer resolution by bringing the dif sites into close proximity, most likely using a KOPS-like mode for directionality of DNA translocation. SftA translocates DNA during septation, while SpolIIIE moves DNA only after division is completed, i.e., through the closed septum. Therefore, dimer resolution is facilitated by SftA before septation is completed, while it is still possible to resolve chromosome dimers between daughter cells, even through two closed membranes, mediated by the two-DNA translocate system in *B. subtilis*. However, the sftA spolIIIE double mutant strain has a more severe phenotype than either single mutant, suggesting considerable overlap in their roles. Because a ΔsftA spolIIIE recC triple mutant shows reduced fitness compared to the ΔrecC single mutant cells, it is clear that the action of DNA translocases is also necessary for a further function, which is the segregation of chromosomes that are still present in the division plane (i.e., during delayed segregation), independently of and in addition to the resolution of chromosome dimers. Since many bacteria contain two DNA translocases, a soluble SftA-like translocase and a FtsK/SpolIIIE like protein, it is likely that the proposed mechanism applies for many bacteria.

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