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Replication Restart after Replication-Transcription Conflicts Requires RecA in *Bacillus subtilis*

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ABSTRACT

Efficient duplication of genomes depends on reactivation of replication forks outside the origin. Replication restart can be facilitated by recombination proteins, especially if single- or double-strand breaks form in the DNA. Each type of DNA break is processed by a distinct pathway, though both depend on the RecA protein. One common obstacle that can stall forks, potentially leading to breaks in the DNA, is transcription. Though replication stalling by transcription is prevalent, the nature of DNA breaks and the prerequisites for replication restart in response to these encounters remain unknown. Here, we used an engineered site-specific replication-transcription conflict to identify and dissect the pathways required for the resolution and restart of replication forks stalled by transcription in *Bacillus subtilis*. We found that RecA, its loader proteins RecO and AddAB, and the Holliday junction resolvase RecU are required for efficient survival and replication restart after conflicts with transcription. Genetic analyses showed that RecO and AddAB act in parallel to facilitate RecA loading at the site of the conflict but that they can each partially compensate for the other's absence. Finally, we found that RecA and either RecO or AddAB are required for the replication restart and helicase loader protein, DnaD, to associate with the engineered conflict region. These results suggest that conflicts can lead to both single-strand gaps and double-strand breaks in the DNA and that RecA loading and Holliday junction resolution are required for replication restart at regions of replication-transcription conflicts.

IMPORTANCE

Head-on conflicts between replication and transcription occur when a gene is expressed from the lagging strand. These encounters stall the replisome and potentially break the DNA. We investigated the necessary mechanisms for *Bacillus subtilis* cells to overcome a site-specific engineered conflict with transcription of a protein-coding gene. We found that the recombination proteins RecO and AddAB both load RecA onto the DNA in response to the head-on conflict. Additionally, RecA loading by one of the two pathways was required for both replication restart and efficient survival of the collision. Our findings suggest that both single-strand gaps and double-strand DNA breaks occur at head-on conflict regions and demonstrate a requirement for recombination to restart replication after collisions with transcription.

Bacterial chromosomal replication initiates from a single origin of replication and proceeds bidirectionally until completion at the terminus. Replication forks regularly encounter obstacles and need to be restarted as they traverse the chromosome (1, 2). The essentiality of restart proteins supports the notion that every single fork initiated from *oriC* will be disrupted at least once, if not more often, before reaching the terminus (3, 4). Transcription frequently impedes replication in bacteria, necessitating numerous factors to resolve conflicts between the two machineries (5). Head-on collisions between replication and transcription, which occur when a gene is carried on the lagging strand, cause mutagenesis, genomic instability, single-stranded DNA (ssDNA) accumulation, and potentially double-strand DNA breaks (6–9).

Previous studies investigating the consequences and coping strategies of cells with transcription from the lagging strand have relied on the use of strains harboring large chromosomal inversions that oriented the highly transcribed, repetitive rRNA gene operons head-on to replication (10–12). In *Escherichia coli*, recombination-mediator proteins were required for efficient survival of rRNA gene inversions, though the repair protein RecA itself did not contribute to viability in these experiments (11, 12). Inversion of the rRNA genes leads to RecA-green fluorescent protein (GFP) focus formation in *Bacillus subtilis* (10). Subsequent studies in *B. subtilis* demonstrated that RecA localizes to loci containing highly expressed head-on protein-coding genes (6). DNA processing by recombination proteins and/or break repair is likely important for replication-transcription conflict resolution. However, the type of DNA damage occurring upon collisions between replication and transcription and the role, if any, that RecA plays at these regions remain elusive.

RecA catalyzes strand invasion as the first step in DNA break repair by homologous recombination (13). Depending on the type of DNA break, RecA is loaded onto the DNA (1) by either the RecFOR or AddAB pathway (14–17). RecFOR recognizes single-

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strand gaps in the DNA (18), likely through an interaction with the single-stranded DNA binding protein (SSB) (19). AddAB, in contrast, processes double-stranded ends to generate 3′ overhangs (20, 21) in a manner analogous to the action of RecBCD in *E. coli* (22). Whether AddAB itself loads RecA onto DNA in *B. subtilis* (an activity mediated by RecB in *E. coli* [23]) is an ongoing topic of debate.

RecA loading and strand invasion lead to the formation of four-way Holliday junction DNA intermediates (24, 25). Cells rely on resolvases, such as the RuvC protein in *E. coli*, to cleave four-way Holliday junction DNA and separate linked sister chromosomes (26–28). RecU, the homologue of *E. coli* RuvC, carries out this function in *B. subtilis* (29–31). When recombination occurs at stalled replication forks, restart proteins associate with resolved recombination intermediates for growth to continue (32–34).

Restart in *B. subtilis* involves an ordered association of essential primosomal proteins, PriA and DnaD, with the DNA, followed by recruitment of helicase loader proteins, DnaB and DnaI. DnaB and DnaI then load the helicase, DnaC, onto the DNA, allowing replication to proceed (35–38). Purified PriA protein can bind both stalled fork structures and D-loops *in vitro*, though it has much higher affinity for the latter (32, 39, 40). Consistent with this, genetic analyses indicated that recombination proteins remodel stalled forks for PriA binding to occur (41). Reconstituted restart reactions *in vitro* demonstrated that both RecBCD and RecOR facilitate restart (42). However, the requirements for PriA binding to DNA *in vivo* remain poorly studied.

We set out to determine if RecA played a role in resolving conflicts between replication and transcription. To address this question, we inserted a single protein-coding gene into the chromosome of *B. subtilis*, oriented either head-on or codirectionally to replication. We find that when the engineered construct is transcribed in the head-on, but not the codirectional, orientation, the presence of at least one RecA loading mediator, RecO or AddAB, and the Holliday junction resolvase, RecU, is required for efficient survival. The transcription and orientation dependence of these phenotypes suggests that replication-transcription conflicts are the underlying cause of the observed survival defects. Epistasis analyses indicate that RecO, AddAB, and RecU act in the same pathway as RecA to promote survival in response to, specifically, head-on transcription. These data suggested that recombination is required for resolution of replication-transcription conflicts. Consistent with this, we find that both AddAB and RecO load RecA onto DNA at the conflict region in a transcription-dependent manner, as indicated by chromatin immunoprecipitations (ChIPs) of RecA. Additionally, we found that RecA is required for the restart protein, DnaD, to associate with the engineered head-on conflict region. Our findings demonstrate that resumption of replication upon conflicts with transcription depends primarily on a classical recombination-mediated and RecA-dependent replication restart pathway in *B. subtilis*.

MATERIALS AND METHODS

Bacterial culture conditions. Cultures were initiated from single-colony isolates grown on solid agar-containing plates supplemented with the appropriate antibiotic. Starter cultures were grown to an optical density (OD) of 0.03 to 0.5 in LB medium at 37°C with vigorous shaking and then diluted back to an OD of 0.05 in an appropriate volume of LB for experimental purposes.

Strain constructions. Gene deletions were introduced by standard transformation techniques as described previously (67). Δ*recO* and Δ*recU* strains were constructed using genomic DNA from deletion mutants obtained from the Bacillus Genetic Stock Center (BGSC). Markerless deletions were made by evicting erythromycin resistance cassettes via flanking *loxP* sites and a plasmid-encoded Cre recombinase (pDR224 carries the *cre* gene and a temperature-sensitive origin of replication), obtained from the BGSC. A detailed list of strains used in this study is in Table S1 in the supplemental material.

ChIPs. Starter LB cultures were initiated from isolated colonies grown on solid agar plates supplemented with the appropriate antibiotic and grown at 37°C, with shaking, to an OD of 0.3 to 0.4. Starter cultures were diluted to an OD of 0.05 in 25 ml LB and grown again at 30°C, with shaking, to an OD of 0.3. Upon reaching an OD of 0.3, cultures were harvested into 0.1% formaldehyde and processed as described previously (43). DnaD IPs were performed essentially as described previously (43). GFP IPs were performed using an anti-GFP antibody purchased from Abcam (ab290) as described by Million-Weaver et al. (6). Quantitative PCRs (qPCRs) to amplify central regions of the *yhaX* and *lacZ* genes were performed as described previously (43), using the oligonucleotides HM192 and -193 (CCGTCTGACCCGATCTTTTA and GTCATGCTGA ATGTCGTGCT), and HM188 and -189 (GGCTTTCGCTACCTGG AGAG and GACGAAGCCGCCCTGTAAAC), respectively.

Microscopy. Microscopy was performed as described previously by Million-Weaver et al. (6). To summarize, cultures were grown in LB supplemented with the appropriate antibiotic at 37° with aeration (260 rpm) to exponential phase (OD = 0.3 to 0.8). Cells were then fixed by formaldehyde treatment, stained with DAPI (4′,6′-diamidino-2-phenylindole), and transferred to agarose pads for visualization by microscopy. DAPI fluorescence and GFP fluorescence were used to quantify total cells and total RecA-GFP foci, respectively. Data analysis was conducted using ImageJ.

Plating efficiency. Cultures were grown in LB supplemented with the appropriate antibiotic at 37° with aeration (260 rpm) to exponential phase (OD = 0.3). Exponential-phase cultures that grew to higher optical densities were diluted to an OD of 0.3. Serial dilutions were plated on LB-agar medium, and total viable cells were quantified for each experimental condition. The plating efficiency ratio for a given genetic background was determined by dividing the viable colonies arising from strains expressing a gene by those arising from strains repressed for transcription of the reporter gene.

RESULTS

RecA, along with RecO and AddAB, promotes survival of cells experiencing severe head-on replication-transcription conflicts. Previous studies in *E. coli* indicated that head-on encounters between replication and transcription from inverted rRNA gene operons caused RecA-independent replication fork reversal (RFR) (11, 12). We wondered if RecA played a role in overcoming conflicts between replication and transcription of a protein-coding gene in *B. subtilis*. There is precedence for this hypothesis, given that RecA-GFP localization has been observed in in *B. subtilis* strains harboring rRNA gene inversions (10) and that RecA association with regions of head-on transcription has been detected by ChIP (6).

To address if and how RecA contributes to survival of replication-transcription collisions, we utilized a previously described site-specific inducible conflict (6, 43). Strains harboring this reporter carry a copy of the *lacZ* gene under the control of the ICEBs1 promoter, P_{xis}, in the head-on orientation with respect to replication at the *thrC* locus of the *B. subtilis* chromosome. In strain backgrounds cured of the ICEBs1 element, the P_{xis} promoter is highly expressed, whereas in the presence of this element, the promoter is tightly repressed (44), leading to minimal expres-

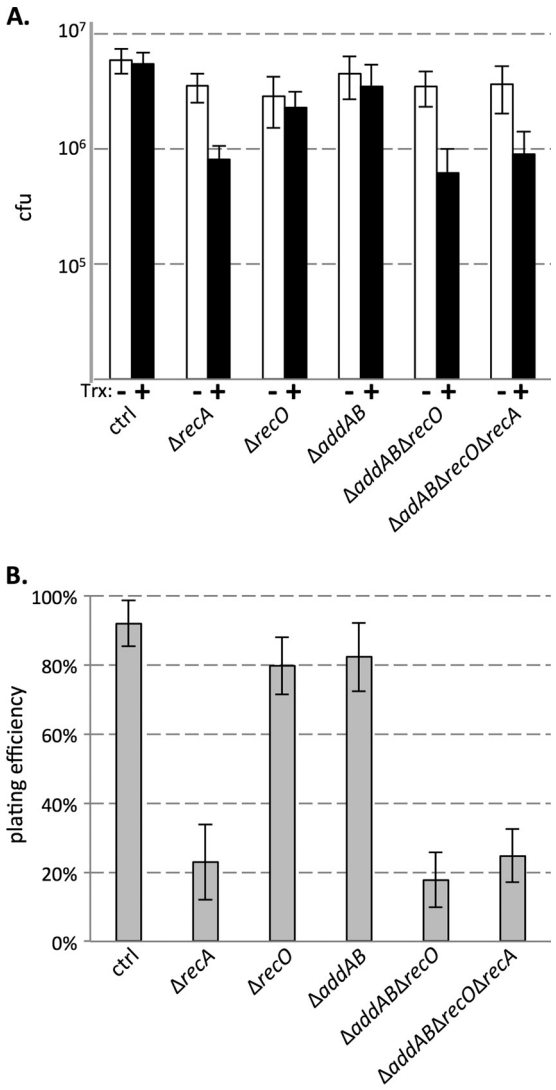

FIG 1 (A) CFU arising from exponentially growing cultures at an OD_{600} of 0.3 were enumerated in the presence (+) and absence (−) of transcription (Trx) from P_{xis}-*lacZ*. (B) Plating efficiencies (transcription-specific survival) were determined by enumerating the ratio of CFU arising from cultures of strains expressing P_{xis}-*lacZ* to that from strains repressed for transcription of the construct in a given mutant background. Data shown are averages from 8 to 12 biological replicates per strain. Error bars represent standard error of the mean (A) or standard deviations (B).

sion of the *lacZ* gene. We grew cultures of *B. subtilis*, either expressing or repressed for transcription of the P_{xis}-*lacZ* gene, to similar optical densities, spotted serial dilutions onto LB agar plates, and enumerated CFU arising under both conditions (Fig. 1A). We then quantified any survival defects associated with deleting genes for homologous recombination under both conditions. To determine the specific contribution of each protein of interest to survival of the highly expressed head-on gene, we took ratios (expressed as plating efficiency percentages) of CFU arising in the presence of transcription of *lacZ* to CFU in strains where *lacZ* was repressed (Fig. 1B).

We found that cells expressing P_{xis}-*lacZ* in otherwise wild-type backgrounds did not display significant reductions in plating efficiency, consistent with the presence of multiple pathways to resolve the negative consequences of head-on collisions between replication and transcription in bacteria (5). Cells lacking *recA* displayed significant survival defects associated with transcription from the *lacZ* gene, leading to a 5-fold reduction in plating efficiency (Fig. 1A and B). Neither deletion of *recO* nor deletion of *addAB* individually caused significant transcription-specific survival defects (Fig. 1B). However, *lacZ* expression significantly impaired survival of cells lacking both *recO* and *addAB* (Fig. 1B). To confirm that the survival defects we observed were specific to head-on conflicts with transcription and not simply *lacZ* expression itself, we also performed plating efficiency assays in strains where the gene was oriented codirectionally with replication (i.e., on the leading strand) (see Fig. S1 in the supplemental material). Although codirectional transcription does impede the replisome (43, 45), these encounters are much less disruptive than when replication and transcription meet head-on. When *lacZ* was expressed from the leading strand, deletion of *recA* or *recO* and *addAB* did not cause any significant reduction in plating efficiencies, arguing against transcription of *lacZ* alone causing survival defects. The orientation specificity implies that head-on conflicts with replication upon *lacZ* transcription necessitate RecA and its loader proteins for efficient survival. The lack of a significant phenotype for either *addAB* or *recO* single mutants, combined with the synergistic effect of combining the two mutations, indicates one of two possibilities: either each individual pathway alone is not important, or one compensates for the other's absence.

We wondered if AddAB and RecO contributed to survival by loading RecA at regions of head-on transcription. To determine whether *recO* and *addAB* act in the same pathway as *recA*, we constructed strains lacking all three genes. We found that combining deletions of *recA* with the *recO* and *addAB* deletions did not lead to any additional survival defects compared to those of the parent strains (Fig. 1A and B). The epistatic relationship of *recA*, *addAB*, and *recO* indicates that both RecO and AddAB facilitate survival of obstacles to replication through RecA.

Both RecO and AddAB load RecA at head-on conflict regions. Two pathways potentially load RecA onto DNA in *B. subtilis:* RecFOR at single-strand gaps or AddAB at double-strand breaks. Previous studies indicated that RecO is required for RecA localization in response to treatment with DNA-damaging agents in cells grown in minimal medium (16). However, the potential contribution of either pathway to RecA loading in cells grown in rich medium without exogenous damage is unclear. The plating efficiency data indicated that both pathways contribute to survival of head-on conflicts through a genetic interaction with RecA. We set out to determine if RecFOR and/or AddAB loads RecA onto the DNA at a specific replication-transcription conflict region in *B. subtilis*.

To determine which RecA loading pathway, RecFOR and/or AddAB, contributed to RecA localization in response to head-on transcription, we first quantified RecA-GFP focus formation in response to transcription of the P_{xis}-*lacZ* reporter using microscopy. In the absence of transcription, we found that RecA-GFP formed foci in roughly 10% of cells, consistent with previous reports (46) (Fig. 2A). In the backgrounds where the *lacZ* reporter was not expressed, cells lacking RecO did not show a reduction in RecA-GFP focus formation, whereas cells without AddAB displayed reduced RecA-GFP localization. When the reporter gene was transcribed, we observed RecA-GFP foci in 20% of cells in otherwise wild-type backgrounds. Deletion of either *recO* or

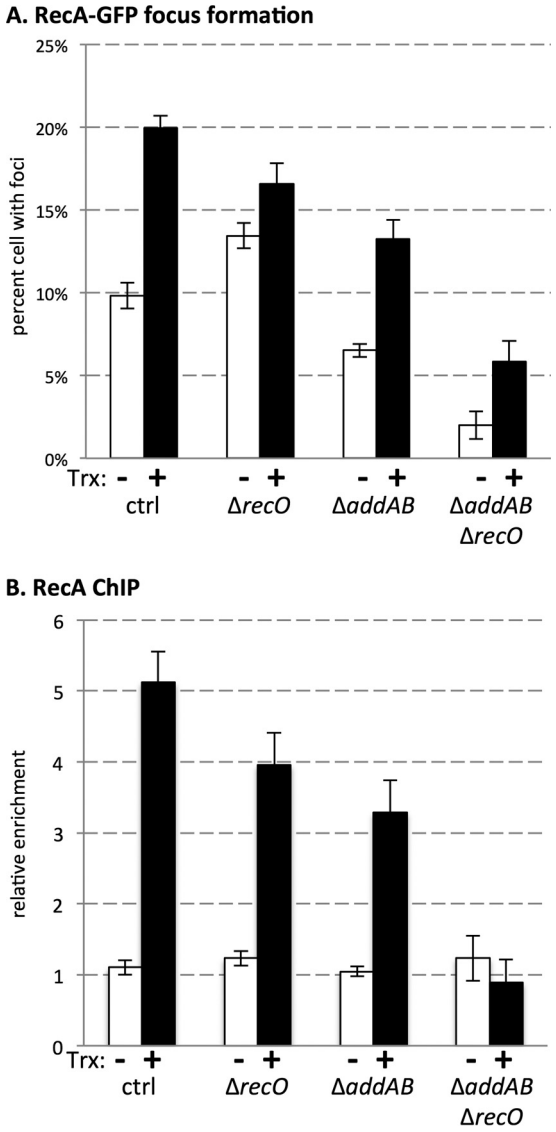

FIG 2 (A) RecA-GFP focus formation was quantified by microscopy in the absence (−) and presence (+) of transcription (Trx) of the P_{xis}-*lacZ* gene. (B) Relative association of RecA with the head-on gene-containing region was measured by ChIP-qPCR in the absence and presence of transcription of the P_{xis}-*lacZ* gene. Data shown represent averages from 6 to 16 biological replicates. Error bars represent standard error of the mean.

addAB led to reduced RecA-GFP localization in cells expressing the *lacZ* gene. Combining the two mutations (*recO addAB* double mutant) had an additive effect (Fig. 2A).

The results of the microscopy experiments suggested that AddAB, and not RecO, is the major mediator required for RecA localization during growth in rich media. Cells lacking RecO did show a modest increase in RecA-GFP localization in the absence of transcription of the reporter, relative to control cells. Therefore, RecO may, to some degree, also load RecA at single-strand gaps under these conditions. The increase observed in RecO-deficient strains probably reflects unresolved single-strand gaps being converted to double-strand breaks due to runoff replication (47).

RecA-GFP focus formation cannot distinguish between general replication stress (i.e., RecA localizing to the DNA elsewhere in the genome) and the specific effects of transcription of the reporter gene. We set out to directly determine the contribution of each pathway to RecA localization specifically at the collision region. To do this, we used ChIPs to measure the relative association of RecA with the replication roadblock, compared to the control locus *yhaX* (other control loci produce similar results to *yhaX* [6]), in the presence and absence of transcription. When the reporter gene was repressed, we did not detect preferential association of RecA with the region in any genetic background. In strains expressing the P_{xis}-*lacZ* construct, we observed a 5-fold enrichment of RecA at the region relative to the control locus (Fig. 2B). RecA association with the region was reduced in strain backgrounds deficient for *recO* or *addAB*. Similar to the results of the microscopy experiments, combining the *recO* and *addAB* deletions had an additive effect; we no longer detected any preferential RecA association at the replication roadblock when *lacZ* was expressed in cells lacking both RecO and AddAB (Fig. 2B). The RecA ChIPs suggest that, consistent with the results of the plating efficiency assays, at least RecO or AddAB is required for RecA to associate with regions of head-on transcription, but one pathway may compensate for the other's absence.

RecU acts in the same pathway as RecO, AddAB, and RecA to help cells survive head-on conflicts. The localization of RecA at the conflict region and contribution to efficient survival suggested that strand invasion at the site of the conflict helped cells tolerate head-on encounters between replication and transcription. Strand invasion by RecA leads to the formation of four-way Holliday junction intermediates, which are cleaved by resolvases to separate the interlinked chromosomes (24, 25). We hypothesized that resolution of recombination intermediates would therefore contribute to cellular survival of replication-transcription conflicts. To test this, we extended our epistasis analysis to determine the effect of the *B. subtilis* Holliday junction resolvase, RecU, which binds Holliday junctions with subnanomolar affinity (31), on plating efficiencies for cells harboring the highly expressed head-on *lacZ* gene.

Deletion of *recU* alone led to reduced plating efficiencies, suggesting that cleavage of four-way junctions contributes to survival of head-on conflicts (Fig. 3). No additional reductions in plating efficiencies were observed when the *recU* and *recA* deletions were combined, indicating that these genes function in the same pathway, likely by forming and resolving four-way junctions, respectively. However, because of the potential limited sensitivity of the plating efficiency assays, we cannot exclude the possibility that RecU performs another RecA-independent DNA processing function at head-on conflict regions.

Because single deletions of *recO* or *addAB* did not cause significant survival defect phenotypes, the genetic interactions between *recU* and either individual pathway cannot be inferred from cellular survival data. However, deleting *recU* in backgrounds lacking both *recO* and *addAB* did not lead to any additional survival defects (Fig. 3). The observed epistasis between all three genes suggests that RecU acts in the same pathway as both RecO and AddAB, as well as RecA, to promote survival of cells upon head-on collisions between replication and transcription. These genetic interactions are consistent with RecU's classically defined function to resolve Holliday junction intermediates arising from homologous recombination.

RecA loading is required for the restart protein, DnaD, to associate with head-on conflict regions. Based on the results pre-

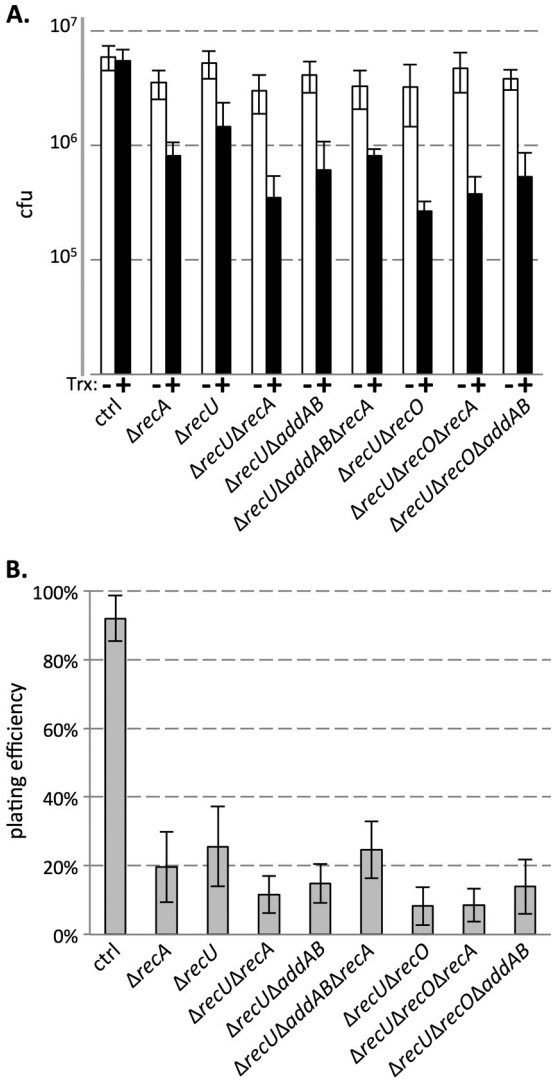

FIG 3 (A) CFU arising from exponentially growing cultures at an OD_{600} of 0.3 were enumerated in the presence (+) and absence (−) of transcription (Trx) from P_{xis}-*lacZ*. (B) Plating efficiencies (transcription-specific survival) were determined by enumerating the ratio of CFU arising from cultures of strains expressing P_{xis}-*lacZ* to that from strains repressed for transcription of the construct in a given mutant background. Data shown are averages from 8 to 14 biological replicates per strain. Error bars represent standard error of the mean (A) or standard deviation (B).

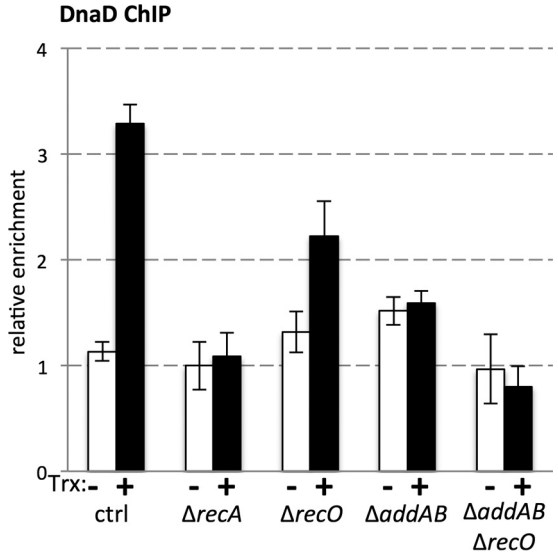

FIG 4 Relative association of DnaD with the head-on gene-containing region was measured by ChIP-qPCR in the absence (−) and presence (+) of transcription (Trx) of the P_{xis}-*lacZ* gene. Data shown represent averages from 12 biological replicates. Error bars represent standard error of the mean.

sented above, we hypothesized that RecA aids replication reactivation in response to conflicts by facilitating the formation of D-loops, which can promote association of replication restart proteins with stalled forks (41, 42). To test this model, we used ChIP to measure the relative association of the restart protein, DnaD, with the roadblock-containing region compared to that for the control locus, *yhaX*, in the presence and absence of transcription in strains deficient for each recombination protein. During primosome assembly, PriA facilitates DnaD loading in order to reload the replicative helicase, DnaC.

Expression of the reporter gene in recombination-proficient backgrounds led to significantly increased relative DnaD association with the *lacZ*-containing region. In strains lacking RecA, we no longer detected preferential DnaD association with the region when the reporter gene was expressed (Fig. 4). Individual deletions of either *recO* or *addAB* reduced DnaD association with the *lacZ* locus (Fig. 4). Similar to the results obtained with the *recA* deletion, in backgrounds lacking both *recO* and *addAB*, we no longer observed preferential DnaD association with the highly expressed head-on gene (Fig. 4).

To rule out the possibility that altered transcription of the P_{xis}-*lacZ* construct in backgrounds lacking *recA* eliminated the obstacle to replication in these strains, we used ChIP to measure the association of RNA polymerase (RNAP) beta subunit (RpoB) with the *lacZ*-containing region with and without transcription from P_{xis}. We found that deletion of *recA* did not alter RNA polymerase occupancy of the reporter gene compared to that observed in wild-type backgrounds (see Fig. S2A in the supplemental material). Furthermore, to rule out the possibility that pleiotropic effects of the *recA* deletion on replication somehow reduced the severity of the effects of conflicts with transcription in these strains, we measured the relative association of the replicative helicase, DnaC, with the region in the presence and absence of transcription. The replicative helicase is expected to localize evenly along the chromosome in an asynchronous population of cells in the absence of specific replication stress. As such, preferential association relative to a control locus probably indicates replication stalling at that region. We found that, as we have reported previously, active transcription from P_{xis} led to a significant increase in the relative association of DnaC with the region (6). This association, however, was not affected by deletion of *recA* (see Fig. S2B in the supplemental material), indicating that severe conflicts between transcription and replication still occur even in *recA* deletion backgrounds and that the absence of RecA specifically inhibits replication restart.

Taken together, these results suggest that a RecA-dependent mechanism is needed for restart proteins to localize to head-on conflict regions. Though RecO and AddAB each partially contrib-

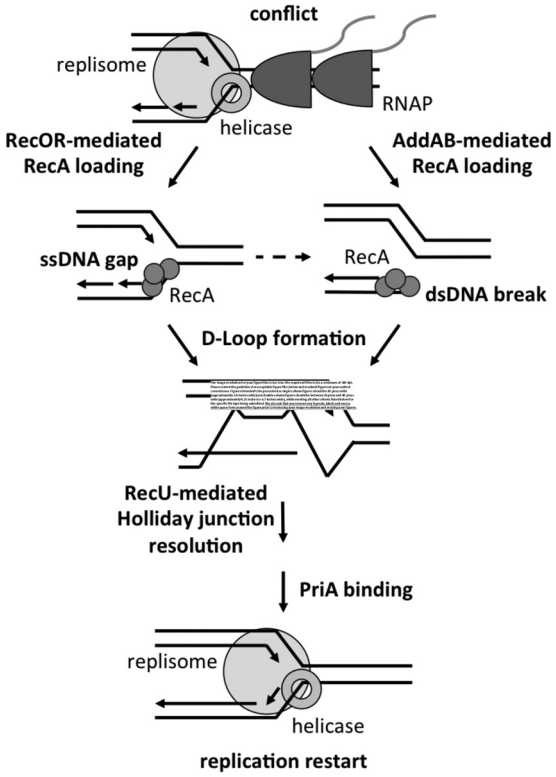

FIG 5 Model for restart upon replication-transcription collision in *B. subtilis*. RecFOR may recognize single-stranded DNA at stalled forks, leading to RecA loading, recombination, and restart. Alternatively, double-strand breaks in the DNA at stalled forks may be processed by AddAB, leading to RecA loading, recombination, and restart.

ute, at least one pathway is necessary for DnaD association with highly expressed head-on genes.

DISCUSSION

Our results demonstrate that RecA contributes to efficient survival of head-on replication-transcription conflicts, likely by promoting replication restart. The plating efficiencies and ChIP data strongly suggest that both single-strand gaps and double-strand breaks occur at head-on conflict regions, both of which are acted upon by RecA (Fig. 5). The survival defects that we observe are modest, indicating that not every conflict causes catastrophic consequences for the cell. However, the requirement for RecA loading for replication restart proteins to associate with the conflict region suggests that when severe collisions do occur, replication requires RecA to proceed. Our results establish RecA-dependent recombination as a prerequisite for the reactivation of replication upon stalling at transcription units in *B. subtilis*.

RecO- and AddAB-mediated conflict resolution. RecA binds single-stranded DNA with 3′ overhangs (48). RecA rapidly polymerizes on DNA to form nucleoprotein filaments, the functional complexes that catalyze strand invasion (49). However, RecA monomers alone have low affinity for Ssb-coated DNA (50). In order for RecA filaments to form, monomers require nucleation onto DNA, a process called RecA loading (51). Two pathways exist for RecA loading in *B. subtilis*, depending on the type of DNA break that occurs.

RecOR loads RecA onto single-stranded DNA in *B. subtilis*. RecO is likely recruited to ssDNA by an interaction with the C-terminal tail of Ssb, as has been demonstrated in *E. coli* (52). AddAB processes double-strand breaks to generate 3′ overhangs (22). Previous work suggested that RecO is required to load RecA onto the DNA regardless of the type of damage, even at double-strand breaks (16). These investigations used RecA-GFP focus formation as a readout for RecA loading in cells growing in minimal medium. We observed RecA-GFP focus formation and DNA association by ChIP even in cells lacking RecO, suggesting that other pathways, such as AddAB, also facilitate loading of RecA at stalled replication forks. The differences between our results and the previous study could arise from the different growth conditions (minimal versus rich medium) or some undetermined cofactor present at replication forks stalled due to transcription that is absent when forks stall in response to chemical DNA damage.

Our results also indicate that one pathway may act in the other's absence, which suggests that AddAB can activate RecA loading independently of RecO. The ability of AddAB to compensate for RecO likely arises because gaps formed at stalled replication forks are converted to double-strand breaks during subsequent rounds of replication (47). RecO may compensate for the absence of AddAB if other nucleases within the cell, such as RecJ in concert with RecQ or RecS (53), resect the double-stranded ends to generate 3′ overhangs for RecA loading. Studies in *Deinococcus radiodurans* have demonstrated that RecFOR can play a predominant role in repairing double-strand breaks (54), suggesting that the absence of AddAB does not necessarily preclude this type of damage repair. Regardless of the mechanism or type of damage, however, in our system, both RecO and AddAB act through RecA.

Sources of single-strand gaps and double-strand breaks at head-on conflicts. The results of the plating efficiency assays and ChIPs showing that both RecO and AddAB contribute to RecA loading and restart suggest that both single-strand gaps and double-strand breaks occur at head-on conflict regions. Our assays lack the sensitivity to directly determine which type of damage predominates; however, it is clear that RecA is important for replication restart at both gaps and breaks. The initial encounter between replication and transcription likely generates a single-strand gap, which would be recognized by RecO. However, because both pathways contribute to tolerating collisions, some double-strand breaks may occur.

We envision three possible scenarios (as illustrated in Fig. 5): direct RecA loading by RecO at single-strand gaps, end processing and RecA loading by AddAB at any breaks that do occur, and, occasionally, gaps being converted into double-strand breaks due to runoff replication.

Single-strand gaps may arise because both RNA polymerase and DNA polymerase unzip the double-helical DNA template, causing negative supercoils and underwound DNA accumulation behind each of the machineries (55–57). Negative supercoiling behind RNAP contributes to R-loop formation, which may maintain one strand of the DNA duplex in a single-stranded state (58). At the site of the collision itself, persistent gaps on the lagging-strand template, such as observed in restart-deficient PriA mutants (59), could necessitate RecFOR-mediated repair.

It is unclear if head-on collisions between replication and transcription directly cause double-strand breaks in the DNA. The torsional strain that accumulates due to excessive positive supercoiling between the two machineries might be sufficient to break

the ester linkages of the sugar-phosphate backbone. However, evidence of chromosome breaks arising directly due to a collision (as opposed to endonucleolytic processing of the DNA in the region) has not been demonstrated.

Any single-strand gaps in the template can be converted into double-strand breaks through replication runoff as forks converge. Direct restart from these structures would likely result in aberrant chromosomes. Therefore, double-strand break repair may be required even if the initial insult to the DNA generated only a single-strand gap or nick.

The role of D-loops in replication restart. PriA allows for origin-independent replication initiation by recruiting helicase loader proteins to the DNA in *B. subtilis*. Purified PriA binds DNA bubble structures with low affinity (dissociation constant [K_d] = 150 nM) yet displays 10-fold-enhanced binding kinetics to D-loop structures (K_d = 15 nM) (40). Previous studies of primosome assembly have relied extensively on reconstituted systems *in vitro* because of the essential nature of most replication restart proteins. Our system allowed us to investigate the requirements for restart *in vivo*. Because RecA and RecA loading were required for DnaD to associate with the conflict-containing region, we hypothesize that a RecA-mediated D loop is required for restart at conflict regions. Additionally, the contribution of RecU to efficient survival suggests a requirement for Holliday junction resolution at head-on conflicts. Consistent with this observation, PriA binds four-way DNA junctions with extremely low affinity (40), and RecU does not cleave D-loops in *B. subtilis* (60). Therefore, although it is speculative, we hypothesize that RecU remodels interlinked chromosomes after strand invasion, generating a D-loop structure which is recognized by PriA.

Differences between replication fork reactivation mechanisms in *E. coli* and *B. subtilis*. Head-on collisions between replication and transcription at inverted rRNA gene operons in *E. coli* are overcome through a RecA-independent replication fork reversal (RFR) mechanism (11, 12). During RFR, the nascent DNA strands dissociate from their templates, regress, and reanneal to form a four-way DNA junction with a double-stranded end. Processing of the regressed fork by RecBCD allows for replication restart subsequent to these encounters. Alternatively, the Holliday junction resolvase, RuvC, may cleave the reversed fork, causing chromosome breakage (61).

Our results suggest that at head-on conflicts, RecA-independent replication fork reversal (RFR) does not occur, at least in *B. subtilis*. In RFR in *E. coli*, RecA is dispensable but RecBCD is essential for viability. Additionally, the RFR model predicts that cleavage of the reversed fork by RuvC (RecU in *Bacillus*) leads to chromosome breakage in RecBCD-deficient cells. Our results clearly demonstrate that RecA associates with head-on conflict regions and establish that RecA loading and Holliday junction resolution reestablish the fork at conflict regions. Additionally, RecA and RecU act in the same pathway to promote conflict survival, suggesting that RecU alone does not play a role in processing the forks such as in the proposed RFR reaction. Nevertheless, we cannot rule out RFR at the conflict region by a RecA-dependent process. Indeed, RecA regresses forks *in vitro* (62), and studies in eukaryotic cells demonstrate that RAD51 promotes fork reversal in response to a broad array of genotoxic stresses (63).

The observed discrepancies between the RecA-independent RFR model and our results could arise from either the nature of the head-on conflict investigated or the model organism studied. The *E. coli* studies were based on rRNA gene inversions, whereas our results rely on a protein-coding gene. rRNA gene operons are repetitive and significantly longer than the single head-on protein-coding gene we used in our study. The impact of gene length on conflict severity and cellular strategies for resolution remains an understudied question. Additionally, rRNA gene transcription is distinct from expression of a protein-coding gene. Antitermination factors such as NusB stabilize RNA polymerase in rRNA genes (64), and high expression from rRNA gene promoters maintains the DNA in these regions in a persistently underwound state behind the transcription apparatus, whereas positive supercoils accumulate ahead of RNAP. Potentially the DNA topology innate to ribosomal DNA contributes to catalyzing fork reversal when operons are inverted head-on to replication.

The fundamental differences between the gammaproteobacteria *E. coli* and the firmicute *B. subtilis* may also account for an absence of detectable RecA-independent RFR in our experiments. *B. subtilis* uses two polymerases for chromosomal replication, DnaE and PolC (65), whereas *E. coli* employs only one (66). Additionally, *E. coli* and *B. subtilis* overcome replication-transcription conflicts by distinct strategies. *E. coli* harbors two accessory helicases, UvrD and Rep, which cooperate to facilitate replication progression across inverted rRNA gene operons, whereas *B. subtilis* appears to harbor only one essential accessory helicase (PcrA), which is probably also important for conflict resolution. *B. subtilis* displays a more significant leading-strand bias (74%) in the organization of its genome than observed in *E. coli* (55%) (5). Finally, inverting the rRNA gene causes cell death in 10% of cells in *B. subtilis*, whereas these genomic rearrangements are not significantly detrimental to survival of *E. coli* cells (10, 11). The results we present here, together with the prior investigations on replication-transcription conflicts in bacteria, highlight the significant differences in resolution mechanisms used by these two model organisms. Therefore, a deep understanding of the mechanisms employed by cells to facilitate accurate and timely DNA replication requires investigations in more than one species.

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