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RadD Contributes to R-Loop Avoidance in Sub-MIC Tobramycin

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ABSTRACT We have previously identified Vibrio cholerae mutants in which the stress response to subinhibitory concentrations of aminoglycoside is altered. One gene identified, VC1636, encodes a putative DNA/RNA helicase, recently named RadD in Escherichia coli. Here we combined extensive genetic characterization and high-throughput approaches in order to identify partners and molecular mechanisms involving RadD. We show that double-strand DNA breaks (DSBs) are formed upon subinhibitory tobramycin treatment in the absence of radD and recBCD and that formation of these DSBs can be overcome by RNase H1 overexpression. Loss of RNase H1, or of the transcription-translation coupling factor EF-P, is lethal in the radD deletion mutant. We propose that R-loops are formed upon sublethal aminoglycoside treatment, leading to the formation of DSBs that can be repaired by the RecBCD homologous recombination pathway, and that RadD counteracts such R-loop accumulation. We discuss how R-loops that can occur upon translation-transcription uncoupling could be the link between tobramycin treatment and DNA break formation.

IMPORTANCE Bacteria frequently encounter low concentrations of antibiotics. Active antibiotics are commonly detected in soil and water at concentrations much below lethal concentration. Although sub-MICs of antibiotics do not kill bacteria, they can have a major impact on bacterial populations by contributing to the development of antibiotic resistance through mutations in originally sensitive bacteria or acquisition of DNA from resistant bacteria. It was shown that concentrations as low as 100-fold below the MIC can actually lead to the selection of antibiotic-resistant cells (3) through the

KEYWORDS DNA repair, R-loop, antibiotic resistance

Bacteria frequently encounter low concentrations (sub-MICs) of antibiotics, and recent studies point to a key role of such concentrations for the genesis of resistance mutants or exogenous resistance acquisition (1). Active antibiotics are commonly detected in soil and water. Concentrations of these antibiotics are well below the MIC but nevertheless can be found at up to several hundred nanograms/liter (2). Although sub-MICs of antibiotics do not kill bacteria, they can have a major impact on bacterial populations. In particular, it was shown that concentrations as low as 100-fold below the MIC can lead to the selection of antibiotic-resistant cells (3) through the
induction of various stress responses (1, 4). SOS is one such response, triggered by a genotoxic alarm signal: single-stranded DNA, which usually results from DNA damage and/or DNA replication blockage (5). We previously found that concentrations as low as 1% of the MIC of various families of antibiotics, even those that do not cause DNA damage, such as aminoglycosides (AG), induce the SOS response in *Vibrio cholerae* and other pathogenic Gram-negative bacteria from different genera (6, 7). Notably, they also increase the mutation frequency and activate the oxidative stress and the RpoS general stress response pathways in both *V. cholerae* and *Escherichia coli*, which can lead to antibiotic resistance (6, 8). Reactive oxygen species (ROS) production was also shown to be central and ultimately to lead to replication and transcription stalling, triggering the SOS pathway (6, 9, 10). Aminoglycosides (such as tobramycin [TOB]) are bactericidal antibiotics that target the ribosome and prevent translation. Sub-MIC aminoglycosides nevertheless trigger the formation of DNA damage, evidenced by induction of SOS (6, 7). A genetic screen developed in our laboratory led to the identification of *V. cholerae* mutants in which the induction of SOS by aminoglycosides is altered (9). A number of the identified genes are involved in replication, recombination, and repair functions, suggesting that sublethal antibiotic stress is sufficient to interfere with the DNA repair and replication machineries and with RNA metabolism. Interestingly, our screen selected for mutants inactivated for the expression of proteins known to destabilize the RNA polymerase (RNAP) complex, such as Mfd. Mfd couples transcription arrests with repair by removing stalled or backtracked RNAP at bulky lesions and recruits the nucleotide excision repair (NER) machinery in a process called transcription-coupled repair (TCR) (11, 12). Stalled elongation complexes can prevent the access of DNA repair enzymes and cause replication-transcription collision. Such complexes also promote formation of structures that constitute further impediments for replication, such as R-loops. Mfd can also dislodge RNAP that pauses at abasic sites due to, for example, base excision repair of oxidative lesions (13). This is of particular interest in the case of Mfd in the response to sub-MIC tobramycin (TOB), as sub-MIC TOB treatment favors incorporation of oxidized bases into DNA (6).

In addition to Mfd, our genetic screen identified the VC1636 gene (9), which encodes a putative DNA/RNA helicase. A homolog of VC1636 was in parallel named RadD in *E. coli* and was shown to carry conserved helicase and DNA binding motifs (14). The closest RadD homolog was found to be the human XPB, a superfamily 2 helicase involved in transcription-coupled repair. *E. coli* and *V. cholerae* RadD proteins are 65% similar (58% identical), including helicase domains. RadD was identified recently by Cox and collaborators in a screen for genes involved in the response to ionizing radiation (15) and was suggested to have a role in DNA double-strand break (DSB) repair in *E. coli* (14, 16). We have identified *V. cholerae* VC1636 RadD as involved in the response to sub-MIC tobramycin stress. VC1636 RadD overexpression, from a high-copy-number plasmid, was able to restore survival of UV in an otherwise UV-sensitive *mfd* mutant (9), leading to the hypothesis that RadD could have a similar function as Mfd in removing stalled RNAP. A subsequent study from the Cox laboratory showed that RadD interacts with the *E. coli* single-stranded DNA binding protein SSB, which stimulates the ATPase activity of RadD (17), and that RadD can bind single-stranded DNA. However, the authors observed no *in vitro* helicase activity.

Here we combined high-throughput approaches and genetic characterization of multiple mutants to address the precise role of the *E. coli* and *V. cholerae* RadD proteins. For the genetic study, we focused on *E. coli*, since previous studies were conducted primarily in *E. coli* and due to the fact that *V. cholerae* mutants with impaired DNA double-strand break repair had poor viability. We show that sub-MIC tobramycin treatment leads to formation of double-strand DNA breaks (DSBs) in the absence of *radD* and that RNase H1 overexpression counteracts such DSB formation. Importantly, we find that the viability of the *radD* deletion mutant strongly relies on RNase H1 function. We further show that RadD directly interacts with the homologous recombination (HR) helicase RecQ. We propose that sublethal aminoglycoside treatment leads
to R-loop-dependent formation of DSBs, which can be repaired by the RecBCD homologous recombination pathway, and that RadD counteracts such R-loop accumulation.

RESULTS

TI-seq identifies rnhA inactivation as highly detrimental in V. cholerae radD. In order to characterize RadD, we addressed its effect in the presence of tobramycin. We adopted a high-throughput transposon insertion sequencing (TI-seq) approach to determine which genes are important in maintaining the cell integrity in the presence of antibiotics at low doses in the radD strain. We chose to perform the TI-seq experiments in Vibrio cholerae, because the changes caused by sub-MIC tobramycin are more marked in this species than in E. coli (6, 18), and radD was identified in the response to TOB in V. cholerae (9). Large transposon inactivation libraries in V. cholerae wild-type (WT) and radD strains were subjected to growth for 16 generations in medium without and with TOB at 50% of the MIC (0.6 μg/ml). After sequencing, insertion detection, mapping, and counts (see Text S1 in the supplemental material), we identified genes where detected insertions had at least a 4-fold increase or decrease in the radD strain and not in the WT after 16 generations (Table 1). We also identified genes with differential detection of insertions at T0 in the radD strain (Table 2). The genes marked with an asterisk in Table 2 were subsequently deleted in our WT and (when possible) in radD V. cholerae strains to confirm the fitness effect revealed by TI-seq (Fig. S1).

Strikingly, the number of detected insertions in rnhA coding for RNase H1 at T0 decreased 4.4-fold in the radD mutant compared to WT (Table 2), and rnhA inactivation was found to be highly detrimental in the radD strain after 16 generations in Mueller-Hinton (MH) (loss of 5.9-fold in radD mutant against 1.7-fold in WT) and even more so in TOB (loss of 13.5-fold in radD mutant against 1.6-fold in WT). We constructed single mutants of rnhA in V. cholerae WT; however, despite our efforts, we could not delete rnhA in the V. cholerae radD mutant (not shown). We took advantage of a thermosensitive plasmid expressing radD+, to construct rnhA deletion mutants at a permissive temperature in V. cholerae WT and radD mutant contexts, but the double mutant strains did not grow upon loss of plasmid at a nonpermissive temperature, suggesting synthetic lethality with radD under these conditions (Fig. S1J). In parallel, we applied a similar strategy in E. coli using P1 transduction of rnhA interrupted by a resistance cassette and found that the E. coli radD rnhA mutant could also not be constructed at a nonpermissive temperature (Fig. S1K). These results show the importance of processing R-loops in the absence of RadD and are consistent with a role of RadD related to R-loop formation/destabilization.

Importance of genes related to DNA metabolism in the V. cholerae radD mutant. At time zero and T16, a large proportion of the genes that are specifically found to be important for the radD strain during antibiotic stress are involved in energy metabolism, general metabolism, and membrane integrity (Tables 1 and 2), among which are two operons that become essential in the radD strain (no insertions detected), the proton-motive-force-dependent tol-pal operon ensuring membrane integrity (19) and the ngr operon involved in oxidative stress (20), suggesting that the radD strain is more sensitive to oxidative and membrane stresses. Another category includes genes related to DNA metabolism (polA, mutT, apaH), suggesting the increased occurrence of DNA damage in the radD strain. Poll (polA) is a DNA polymerase responsible for stripping RNA primers during lagging-strand replication but is also pivotal in various DNA repair pathways in E. coli (21, 22). ApaH is involved in detoxification of toxic DNA bases (23) and resistance to stress (24), and MutT limits incorporation of potentially mutagenic oxidized guanine residues into DNA (25). Interestingly, mutT inactivation detection decreased 13.6-fold in the TI-seq experiment in radD TOB compared with only 2.4-fold in WT TOB. Moreover, as described above for the rnhA radD synthetic lethality, the polA radD double mutant could also not be obtained in V. cholerae using the same strategy (not shown). The identification of these genes points to amplified DNA damage in the absence of radD and suggests that the radD strain is somehow less tolerant to oxidative variations (even in the absence of TOB) and could have difficulties
coping with the incorporation of modified nucleotides in DNA (or RNA) compared with the WT. These results do not exclude, however, the occurrence of such stress in the WT context upon TOB treatment, consistent with our previous results showing the importance of MutT in response to sub-MIC tobramycin in *V. cholerae* (6). Subsequent growth assays indeed show that deletion of *mutT* causes a slight growth defect in both WT and *radD* strains (Fig. S1M). Several gene inactivations whose detections increase in *radD* were also identified (Tables 1 and 2). We constructed simple and *radD* double deletion mutants for several such genes, i.e., *yycC*, putative tRNA pseudouridine synthase; RdgC, which inhibits RecA-mediated strand exchange *in vitro* (26); and YeBG, belonging to the SOS regulon and DSB processing pathways (27), but we observed no significant effect on growth in either MH or TOB (not shown), although they appear to slightly increase the MIC of TOB (Fig. S1L). Further study needs to be carried out to elucidate the interplay between RadD and these factors. Finally, since RadD was previously suggested to be involved in DSB repair, we expected to find *recB* inactivation as detrimental in the *radD* context, but the stringency of our analysis did not show the loss of detected

### TABLE 1 Genes where insertions are specifically lost or enriched in the *radD* strain after evolution in MH or TOB

| Decrease or increasea and antibiotic | Gene ID | Fold change for *T*16 vs *T*0 (P value) for strain: |
|-------------------------------------|---------|---------------------------------------------------|
| Decrease | | MH *radD* strain* | MH WTb | TOB *radD* strainc | TOB WTd |
| No antibiotic | VC1835 pol | Outer membrane integrity | No reads (0) | −1.8 |
| VC1837* tolA | Outer membrane integrity | No reads (0) | 8.3 |
| VC1838 tolR | Outer membrane integrity | No reads (0) | 5.9 |
| VC1839 tolQ | Outer membrane integrity | No reads (0) | −1.2 |
| VC2291 ngrE | Iron and oxidative stress | No reads (0) | 6.2 |
| VC2292 ngrD | Iron and oxidative stress | No reads (0) | 1.1 |
| VC2293 ngrC | Iron and oxidative stress | No reads (0) | 1.2 |
| VC2294 ngrB | Iron and oxidative stress | No reads (0) | 5.1 |
| VCA0897 pgl | Pentose phosphate pathway | −26.3 (0.009) | 2.1 |
| VCA0609 Unknown | −23.4 (1.60E−15) | 2.9 |
| VCA0634 Putative tRNA modification | −9.3 (0.011) | 1.5 |
| VCA2517 Putative ABC-type transport | −6.7 (1.00E−08) | 1.5 |
| VC2234* mhA | RNase H1, R-loop degradation | −5.9 (0.014) | −1.7 |
| VCA1575 Unknown | −4.1 (4.50E−07) | −1.7 |
| TOB, 50% MIC | VC1948 Unknown | 1.6 | −1.5 | −30.9 (0.001) | 1.2 |
| VC0678 hlyU | Transcriptional regulator | −2.9 | −3.2 | −16.8 (0.001) | 1.3 |
| ncrRNA235 Noncoding RNA | −1.3 | 2 | −16.2 (0.006) | −1.8 |
| VC2392* mutT | Nucleotide detoxification | 1 | −1.8 | −13.6 (8.70E−05) | −2.4 |
| VC2234* mhA | R-loop degradation | −5.9 | −1.7 | −13.5 (0.002) | −1.6 |
| VCA0569 vrrE | Unknown | −1.8 | 1.8 | −10.3 (1.90E−04) | 1.2 |
| VC2718 bioH | Metabolism | 1.9 | 1.2 | −8.7 (1.50E−04) | −2.2 |
| VC1759 Prophage integrase | −1.1 | 1.3 | −8 (0.001) | 1.6 |
| VCA0032 Unknown | −2.8 | −1.4 | −5.6 (2.20E−04) | −1.5 |
| VCA0741 Unknown | 1.8 | 2.2 | −5 (0.008) | −1.3 |
| VCA0654 scrR | Carbohydrate metabolism | −1.6 | 2.1 | −4.9 (5.80E−05) | −1.2 |
| VC0099 glpG | Protease | 1.3 | −1.6 | −4.8 (3.70E−06) | −1.5 |
| VC1824 Carbohydrate metabolism | −1.3 | −1.7 | −4.5 (0.004) | −1.1 |
| VCA0608 yjjG | Nucleotide detoxification | −1.2 | 2.1 | −4.2 (1.90E−05) | −1 |
| VCA0501 Unknown | 1.3 | 1.5 | −4.1 (2.60E−11) | 1.9 |
| Increase | No antibiotic | VC0887* yacC | Pseudouridyl synthase (Hyp) | 8.3 (0.045) | 2.9 |
| VC0330 rsd | Putative transcription factor | 6.5 (0.003) | 1.1 |
| VC1167 tdk | Pyrimidine metabolism | 4.7 (7.20E−09) | 2.7 |
| TOB, 50% MIC | VC1262 Putative methyltransferase | −1.1 | 1.3 | 6.4 (3.00E−05) | 1.7 |
| VCA1150 Unknown | 2.4 | 1.2 | 4.8 (1.00E−04) | 2.1 |

aAverage insertions detected in MH *radD* strain at *T*16 compared to *radD* strain at *T*0; all numbers express fold changes.

bInsertions in MH WT at *T*18 compared to WT *T*0.

cAverage insertions detected in TOB *radD* strain at *T*16 compared to *radD* strain at *T*0.

dAverage insertions detected in TOB WT at *T*16 compared to WT *T*0. Genes with at least 4-fold changes are shown. Deletions for genes marked with an asterisk were constructed in *V. cholerae* WT and *radD* strains.

In *radD* strain but not WT at time *T*0.
insertions in recB as statistically significant. This is due to the low number of initial insertions in the recB gene in both WT and radD contexts and further decreased detections after 16 generations in TOB. However, when we specifically look at the faith of detected insertions after 16 generations in the absence of TOB, the number of reads decreases 6-fold in the radD mutant but not in the WT, supporting the hypothesis that DSB repair is important in the absence of radD.

**Coupling of transcription and translation is critical in radD mutant and in sub-MIC TOB.** Another category of genes whose inactivation affects growth of the radD strain relates to translation, particularly ribosome biogenesis and stability factors (such as KsgA and EF-P, a translation-transcription coupling factor (Table 2). Insertion counts decreased 5.4-fold for efp in the radD mutant at time zero compared to WT. We found that the deletion of efp affects the growth of radD even in the absence of antibiotics (Fig. S1A and B), suggesting that the coupling of transcription and translation is important in this mutant. Moreover, we observe that deletion of efp is lethal in TOB at 50% of the MIC, even in the radD\(^+\) context, highlighting the need for translation-transcription coupling upon exposure to sub-MIC TOB (Fig. S1A to C).

### Table 2: Genes with differential insertions at T₀ in radD strain

| Change in no. of insertions in radD mutant at T₀ (compared to WT) and gene type | Gene ID | Locus tag | Name | Role | WT\(^a\) | radD mutant | Fold change of no. of insertions in radD strain compared to WT, both at T₀ | P value |
|---|---|---|---|---|---|---|---|---|
| Decrease | DNA/RNA metabolism | VC0108 | polA | DNA replication/repair | 109 | 6 | 19.5 | 0.019 |
| | VC0441\(^d\) | apaH | Purine metabolism | 400 | 90 | 4.9 | 0.007 |
| | VC2392\(^c\) | mutT | Nucleotide detoxification | 387 | 81 | 4.8 | 0.049 |
| | VC2234\(^c\) | rnhA | RNase H1, R-loop degradation | 245 | 55 | 4.4 | 0.010 |
| Translation | VC0443\(^d\) | ksgA | rRNA modification | 215 | 13 | 16.3 | 0.019 |
| | VC2679 | rpmE | Ribosomal protein | 513 | 39 | 13.3 | 0.075 |
| | VC0582 | rsmI | rRNA modification | 387 | 44 | 8.7 | 0.019 |
| | VC2660* | efp | Translation elongation factor | 222 | 41 | 5.4 | 0.019 |
| Other | VC0556* | gshA | Thiol redox system | 134 | 2 | 67.0 | 0.040 |
| | VC0824 | tpx | Thiol redox system | 560 | 85 | 6.6 | 0.022 |
| | VC2381 | btfF | Vitamin B12 ABC transporter | 330 | 67 | 4.9 | 0.016 |
| | VC2288 | nqrM | Energy metabolism | 314 | 69 | 4.5 | 0.016 |
| | VC0240 | rfaD | LPS | 223 | 4 | 59.4 | 0.013 |
| | VC1215 | pgsA | Cell membrane integrity | 208 | 7 | 30.4 | 0.042 |
| | VC2156 | nlpC | Outer membrane integrity | 414 | 46 | 9.0 | 0.022 |
| | VC1044 | Unknown | 622 | 53 | 11.7 | 0.038 |
| | VC0300 | Unknown | 271 | 16 | 16.9 | 0.015 |
| | VC0911 | treA | Trehalose metabolism | 331 | 31 | 10.8 | 0.032 |
| | VC2669 | Tyrosine metabolism | 462 | 78 | 5.9 | 0.000 |
| | VC0395 | gtaB | Carbohydrate metabolism | 446 | 53 | 8.4 | 0.043 |
| | VC0964 | crr | Carbohydrate metabolism | 341 | 72 | 4.7 | 0.004 |
| | VC0721 | pstS | Phosphate ABC transporter | 289 | 71 | 4.1 | 0.047 |
| | VC1802 | Unknown | 516 | 19 | 27.5 | 0.011 |
| | VC1810 | Unknown | 508 | 31 | 16.5 | 0.040 |
| Increase | VC2326* | yebG | dsDNA-binding SOS protein | 19 | 136 | 7.2 | 0.05 |
| | VC0718 | rdpC | Electron transport | 26 | 159 | 6.2 | 0.03 |
| | VC0718* | rdgC | NAP | 34 | 199 | 5.9 | 0.03 |
| | VC1693 | torC | Energy metabolism | 59 | 255 | 4.3 | 0.05 |

\(^a\)Normalized average reads.

\(^b\)Values in boldface are decreases; values in italic are increases. These numbers correspond to fold changes calculated with average insertions that included decimals. Genes with at least 4-fold differences are shown.

\(^c\)Deletions for genes marked with an asterisk were constructed (when possible) in V. cholerae WT and radD strains. ksgA mutants could not be obtained.

\(^d\)ksgA and apaH are in the same operon.

Abbreviations: LPS, lipopolysaccharide; dsDNA, double-stranded DNA; NAP, nucleotide-associated protein.
RadD directly interacts with RecQ. In parallel, in order to identify protein partners of RadD, we performed a tandem affinity purification assay (TAP-tag [Text S1] [28, 29]), under conditions with and without antibiotic stress in *V. cholerae* (data not shown). Selected proteins were then tested by yeast two-hybrid assay (30), among which was RecQ helicase. RecQ, together with SSB, has been previously identified in a TAP-tag assay with *E. coli* RadD (17), but RecQ was suggested to be detected because of a coassociation with SSB. We observed here strong direct interaction between *V. cholerae* RadD and RecQ (Fig. S2). On the other hand, no interaction was observed between RadD and the RNA polymerase subunits RpoB/C (not shown).

The RecBCD double-strand break homologous recombination repair pathway is important in the response to tobramycin in the absence of radD. In parallel to the high-throughput approach, we undertook an extensive genetic study in *E. coli*, due to the fact that *V. cholerae* mutants with impaired DNA repair had poor viability. To analyze the response of different mutants to TOB, we assayed growth in TOB at 50% of the MIC (0.25 μg/ml for *E. coli*). Deletion of *radD* alone conferred no growth defect (Fig. 1A and Table S2). In order to understand which pathways could be linked with the function of RadD, we inactivated several genes related to DNA stress and repair pathways in *E. coli*: *recB* (HR, double-strand break repair), *recF* (HR, single-strand gap repair), *uvrA* (NER), *dinB* (translesion synthesis), and *rep* and *dinG*, which are accessory replicative helicases that clear DNA from roadblocks (31). We then tested growth of single and double *E. coli* mutants in MH and TOB. No negative effect was observed for deletion of *uvrA*, *dinG*, *rep*, and *dinB* in the *radD* context, in MH, or in TOB (Fig. S3), consistent with TI-seq data. This suggests that replication in the *radD* mutant is not impaired by roadblocks and bulky complexes or lesions and that NER is not needed. On the other hand, inactivation of *recB* (Fig. 1B) but not *recF* (Fig. 1C) was observed to be detrimental in the *radD* mutant. This points to DSB formation in the *radD* mutant in the presence of TOB, and even in MH without antibiotic, thus requiring RecBCD homologous recombination.

DSB formation in *E. coli recB* and *recB radD* strains was quantified using a fluorescent terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. In this system, double-strand ends, including those generated by DSBs, are fluorescently labeled and quantified by flow cytometry. We used *recB* derivatives for this assay, so that DSBs that are formed cannot be repaired and thus can be accurately measured. Figure 2A (and Table S2 for statistical significance) shows that fluorescence is increased in the *recB radD* strain compared to the *recB* single mutant in TOB. These results are consistent with the hypothesis that DSBs are formed in the *radD* mutant in sub-MIC TOB and that these breaks are repaired by the RecBCD HR pathway.

R-loops are responsible for part of the DSBs formed in the absence of radD in TOB. RadD had been reported previously to be involved in DSB repair (14, 16), but no molecular mechanism was proposed. Having identified *radD* in a stalled-transcription screen (9), and based on our TI-seq data identifying *rnhA* deletion as detrimental in the
radD mutant, we were in a position to ask the question of whether DSBs formed in the radD mutant could arise from R-loops. Indeed, R-loops are frequently formed under conditions where RNAP stalls (32, 33) and can be at the origin of DSB formation when they are not degraded by RNase H1 (rnhA) (32, 34).

In order to test this hypothesis, we first undertook the construction of various rnhA mutant derivatives in E. coli. However, as previously described (33, 35–38), all the strains carrying rnhA inactivation quickly accumulated suppressor mutations. A second strategy was used to look for the phenotype of RNase H1 overexpression in our different mutants: we compared growth of E. coli recB and recB radD strains transformed with a plasmid overexpressing RNase H1 or with an empty plasmid. We also tested isogenic recB/H11001 strains. No effect of RNase H overexpression was observed in the recB/H11001 context for the WT and radD mutant (Fig. S4). In the recB-deficient context, although we observed a slight improvement by RNase H1 overexpression on growth of the recB strain in TOB (Fig. 3A), the effect was even more marked in the recB radD mutant, where pRnhA/H11001 significantly improved growth (Fig. 3B).

We quantified DSB formation in the presence of the RNase H1-overexpressing plasmid in E. coli (Fig. 2B). Interestingly, introduction of the empty plasmid led to slightly higher DSB levels in the recB mutant in TOB. In the recB radD context with empty plasmid, DSB levels were increased compared to the recB strain, which was
consistent with what was observed in the plasmidless assay (Fig. 2A). When pRnhA was introduced in the recB radD context, the DSB levels decreased compared to the isogenic strain with empty plasmid in TOB. These results suggest that the overexpression of RNase H1 relieves DSBs that are formed in the absence of radD in TOB and that R-loop formation at least partly accounts for the viability loss of the radD mutant, suggesting that radD could have a role in the avoidance/destabilization of R-loops.

RadD is involved in R-loop degradation/limitation in vivo. We were unable to test direct unwinding of RNA-DNA hybrids in vitro despite our efforts to purify an active form of V. cholerae RadD. In order to address whether R-loop formation is increased in vivo in the absence of RadD, we used the properties of the dnaA(Ts) rnhA mutant where stable DNA replication occurs at R-loops throughout the chromosome (39). As DnaA is essential for priming of chromosome replication in E. coli, the dnaA(Ts) thermosensitive mutant cannot grow at 42°C. Inactivation of RNase H1 (rnhA) in the dnaA(Ts) mutant restores viability because of increased formation of R-loops, which can prime replication initiation. We hypothesized that if R-loop formation is increased in the radD mutant, then the dnaA(Ts) radD strain would also grow at 42°C. Figure 4 shows that although all mutants have similar growth profiles at the permissive temperature (30°C, Fig. 4A), only the inactivation of rnhA restores viability at 42°C, and not radD (Fig. 4B). This means that the number of R-loops that are formed upon radD deletion is not increased to levels sufficient to promote stable replication in the dnaA(Ts) background. However, when these mutant strains were grown at 30°C and then restreaked at 42°C, we observed growth of several colonies in the dnaA(Ts) radD strain but not in the dnaA(Ts) strain. We quantified the appearance of these colonies by plating the cultures at 42°C and observed that there is an increase of CFU from $3 \times 10^{-6}$ in the dnaA(Ts) strain to $2 \times 10^{-4}$ in the dnaA(Ts) radD strain (Fig. 4C). Since spontaneous mutation frequencies were not increased in the radD or radD dnaA(Ts) strain compared to isogenic radD$^+$ strains (data not shown), this ~100-fold increase of spontaneously
growing colonies was unexpected. These CFU could appear due to genetic suppression mutations or to stochastic phenotypic variation. When we restreaked these CFU at 42°C, only 37% to 50% grew again, independently of the fact that the strain was deleted or not for radD (Fig. 4C). These results suggest that more R-loops are formed stochastically in the radD strain and that this phenotype cannot be inherited, meaning that at least half of the obtained CFU are not genetic suppressors. A major difference between planktonic and colony growth is oxygen availability. One possible explanation for the growth of the radD dnaA(Ts) strain in solid and not liquid medium could be that the strain could be particularly sensitive to oxygen and therefore will grow only in colonies that are under mostly anaerobic growth. Using a high-copy-number plasmid (~100 copies), we next addressed whether overexpression of RadD has a negative effect on R-loop formation. The dnaA(Ts) rnhA strain with empty plasmid grows at 42°C (Fig. 4D). Reintroduction of rnhA in trans prevents growth as expected, and so does overexpression of E. coli radD and V. cholerae radD. In order to ascertain that growth prevention is not due to protein overexpression, we also expressed a V. cholerae protein with a putative RNase function (VCA498) and found no effect on growth. Altogether, these results show that RadD overexpression has a negative effect on R-loop formation. R-loop formation is under some conditions related to DNA superhelicity levels. We tested in vivo whether supercoiling levels could be different in the radD mutant using an assay developed previously in the laboratory (40) and found that RadD has an impact on DNA topology (Fig. S5); however, chloroquine gels to test plasmid supercoiling in the presence or absence of RadD did not yield conclusive results regarding an effect of RadD on topology in this assay (not shown).

DISCUSSION

We show here that in the absence of radD, V. cholerae relies on various factors, such as RNase H1, for efficient response to sub-MIC TOB. The results also highlight the fact that the presence of sub-MIC TOB leads to DSBs, at least partly through R-loop formation, explaining the need for DSB repair in the absence of RadD.

In previous studies, the E. coli radD single mutant showed only a very small defect in survival of UV irradiation compared to the WT strain (14), unlike the UV-sensitive mfd mutant (41). When we further addressed the role of RadD in the response to UV damage, and a possible link with Mfd, the radD mfd double mutant showed higher UV sensitivity than the mfd single mutant, suggesting that RadD and Mfd may have overlapping functions in response to UV irradiation (not shown). The absence of these factors affects also the response to sub-MIC TOB, pointing to impaired transcription.

The link between transcription impediments, R-loop formation, and DSBs has been described in prokaryotes and eukaryotes. It is known that R-loops accumulate at stalled transcription elongation complexes (32, 33) and in the absence of effective transcription termination (42). In human cells, it was shown that R-loops provoke DSB formation by interfering with replication (43–45). In bacteria, replication-transcription collisions are known to lead to genomic instability and breaks (32, 46). Previous work has established that R-loops generate DSBs because they constitute replication blocks and that RNAP backtracking is an important factor potentiating the formation of such R-loop extensions and DSBs (32). The fact that we did not see any effect of the inactivation of replicative helicases such as Rep or DinG in the radD strain suggests that the absence of radD does not cause replication blocks. However, R-loop-dependent genome instability is not necessarily due to replication blocks. In a recent study, it was shown that R-loop-dependent DSB formation in E. coli was due not to replication impairment but to formation of RNA gaps at R-loops (RNA-DNA junctions at arrays of R-loops), which lead to chromosomal DSBs (34). Importantly, overexpression of RNase H1 and active antibacktracking mechanisms suppress such DSB accumulation in E. coli (32). Another HR helicase proposed to prevent R-loop formation is RecG (47). Deletion of recG is coletal with rnhA and promotes stable DNA replication. However, our TI-seq data predict no co lethality of recG and radD, as insertions in recG are detected at equivalent levels in WT and radD strains in the presence or absence of TOB. On the
other hand, the *E. coli* radD recG mutant was previously studied (14) and the authors found that the strain rapidly accumulates suppressors and proposed that this could be due to a DSB repair defect. Here, RecG does not appear to be important in the absence of RadD, but one cannot rule out the possibility that RecG and RadD may have overlapping functions against R-loops. DSB formation could also be linked to DNA structures formed upon inappropriate R-loop processing in a *radD* mutant. R-loops can also interfere with DNA damage repair. It was shown in yeast that RNase H1 is important at DSBs against R-loops which otherwise impair recruitment of RPA (replication protein A, the SSB orthologue; SSB) and subsequent access of HR proteins to DSBs (48). Along the same line, a recent study showed that the human transcription-coupled repair protein CSB is recruited to R-loops induced by reactive oxygen species (ROS) at transcribed sites to initiate repair by HR (49).

How RadD counteracts/reduces R-loop formation is unclear. One possibility is through an effect on DNA supercoiling, which is linked to R-loop formation. In *E. coli*, TopoI is known to interact with RNAP and reduce R-loops (50) and its depletion leads to negative supercoiling behind the transcribing RNAP, enhancing R-loop formation (36). We observed that RadD has an impact on DNA topology, but this effect can also be indirect.

On the other hand, we know that RadD interacts with SSB (17) and with RecQ (this study). One possible hypothesis for RadD action would thus be that RadD together with RecQ could directly destabilize/unwind R-loops and recruit SSB at DSBs. SSB stimulates the activity of RNase H1 (51) and enhances the DNA helicase activity of RecQ in *E. coli* (52) and human cells (53, 54). RecQ can impact R-loop formation (55, 56) through effects on replisome stability at transcription-replication conflicts or direct unwinding of R-loops (57) or through changes in superhelicity (57–59). *topA* and *recQ* mutant backgrounds could be used in future work to more clearly define the role of RadD.

Interestingly, it was shown that the eukaryotic RecQ5 associates with RNAP and enforces the stability of ribosomal DNA arrays (60). Translating ribosomes also inhibit DSB formation at transcription sites (33). Indeed, slowing or blocking translation leads to DSB formation in the absence of R-loop repair (34). Thus, a role for RadD-RecQ can also be envisaged at the translation-transcription level. We can speculate that RadD could be important under conditions where translation is slow/impaired for the following reasons: (i) RadD is involved in the response to TOB, which interferes with translation; (ii) slow translation can promote R-loop formation; and (iii) our TI-seq experiment identified several translation-related factors that are important for the fitness of the *radD* mutant (Tables 1 and 2), namely, EF-P and KsgA. KsgA is a ribosome biogenesis and stability factor. EF-P counteracts ribosome pausing and maintains transcription-translation coupling (61).

Coupling of transcription and translation reduces R-loop formation in bacteria and subsequent DSB formation, as a newly transcribed RNA can be bound immediately by ribosomes (62). In *E. coli*, RNA polymerase also directly binds to ribosomal subunits in vivo, which could facilitate coordination of transcription and translation (63). In fact, the rate of transcription was shown to be controlled by the rate of translation (64). Slow translation leads to RNAP backtracking (65, 66). Accordingly, translation prevents transcription-related formation of DSBs (32). Transcription-translation coupling can be disrupted upon ribosome stalling (in the *efp* mutant or when aminocyl-tRNAs are limiting [67, 68]). Notably, the EF-P transcription-translation coupling factor was identified as a suppressor of the growth defect in the *rnhA topA* mutant (69), suggesting that translation can also counteract R-loops that are formed due to accumulated negative supercoiling. Another example is the *rep uvrD* mutant, which is lethal due to conflicts between replication and transcription elongation complexes. This lethality can be suppressed by *rpo*- alleles destabilizing RNAP (31) but also by mutations in EF-P (70). One hypothesis regarding the anti-R-loop action of RadD could therefore be at the level of translation-transcription coupling. Under this model, the involvement of RadD in the response to TOB effects of ribosome progression is coherent.

Here, we have initially addressed the function of RadD in response to sub-MIC
tobramycin. In the light of our results and the discussion above, we propose that TOB, even at sub-MIC levels, impedes translation, which primes defects in transcription, thus enhancing R-loops/R-lesions at transcription sites, causing DSBs that are repaired by the RecBCD HR pathway. SOS is indeed triggered here by DSB repair as observed previously (7, 9). We hypothesize that RadD, together with RecQ, acts either at the level of translation-transcription coupling for the avoidance of R-loop formation or directly at the R-loop before DSBs arise (Fig. 5). Further study is needed to unravel the exact mechanism of action of RadD on R-loops. Interestingly, the radD gene is located next to the rsuA gene putatively involved in ribosome assembly. Although we found no direct interaction between the RadD and RsuA proteins (two-hybrid data, not shown), we observe that the synteny is conserved among many gammaproteobacterial genera, such as Escherichia, Klebsiella, Salmonella, Serratia, and Shewanella. Finally, sub-MIC TOB may not affect all ribosomes equally, leading to heterogeneity of responses within a clonal population. Single-cell approaches (such as microfluidics) would be complementary and suitable in future research to compare behaviors and responses at both subpopulation and whole-population levels.

MATERIALS AND METHODS

MH medium was used for the study of the effect of sub-MIC tobramycin. TOB was aliquoted and stored at a 10-mg/ml concentration at −20°C. A fresh aliquot was used for each experiment.

Plasmids, strains, and oligonucleotides used in this study and their constructions are listed in Table S1 in the supplemental material. E. coli mutants were constructed by P1 transduction, and V. cholerae mutants were constructed by homologous recombination after natural transformation or with a conjugal suicide plasmid (pMP7/pWS7848) as described previously (6, 71, 72).

Growth kinetics were performed from overnight cultures from single colonies, using the Tecan Infinite plate reader on 96-well plates for 10 h at 37°C with shaking. OD600 was measured every 5 min.

Growth curves (CFU counts) of the dnaA(Ts) derivatives were performed as previously described (9). Double-strand break quantification was performed using the Promega fluorometric TUNEL system. An overnight culture was diluted 100× in MH with or without 0.2 μg/ml TOB and grown to an OD600 of 1. Carbenicillin (100 μg/ml) was added to the growth medium for plasmid-carrying strains. One milliliter (3 × 106 to 5 × 106 cells) was centrifuged at 2,000 rpm for 15 min at 4°C, washed twice with cold PBS, and resuspended in 500 μl PBS. Cells were fixed with 5 ml 1% methanol-free formaldehyde on ice for 20 min, washed twice with cold PBS, and permeabilized overnight with 5 ml ice-cold 70% ethanol. Cells were then washed twice with PBS and stained according to the manufacturer’s recommendations. Green fluorescence was measured on a Miltenyi MACSQuant flow cytometer.

Transposon insertion sequencing libraries were prepared as previously described (9, 73) to achieve a library size of 600,000 clones and subjected to passaging in MH and MH with TOB at 0.5 μg/ml for 16 generations. Sequencing and analysis are described in detail in the supplemental material (see Text S1). Briefly, sequencing libraries were prepared using Agilent’s Sureselect XT2 kit with custom RNA baits designed to hybridize the extremities of the Mariner transposon. Illumina paired-end sequencing technology was used, producing 2- by 125-bp-long reads. Reads were filtered through transposon mapping to ensure the presence of an informative transposon/genome junction as described previously.
(74). Expansion or decrease of fitness of mutants was calculated in fold change with normalized insertion numbers (75). Baggerly’s test on proportions (76) was used to determine statistical significance, and Bonferroni correction was applied for multiple testing.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01173-19.

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