Transcription-coupled DNA repair underlies variation in persister awakening and the emergence of resistance

Wilmaerts Dorien^{a,b}, Focant Charline^b, Matthay Paul^{a,b}, Michiels Jan^{a,b}

^aCenter for Microbiology, VIB, Leuven, 3001, Belgium

^bCentre of Microbial and Plant Genetics, KU Leuven, Leuven, 3001, Belgium

Correspondence: jan.michiels@kuleuven.be

Summary

Persisters constitute a population of temporarily antibiotic-tolerant variants in an isogenic bacterial population and are considered an important cause of relapsing infections. It is currently unclear how cellular damage inflicted by antibiotic action is reversed upon persister state exit and how this relates to antibiotic resistance development. We demonstrate that persisters, upon fluoroquinolone treatment, accumulate oxidative damage which is repaired through nucleotide excision repair. Detection of the damage occurs via transcription-coupled repair using UvrD-mediated backtracking or Mfd-mediated displacement of the RNA polymerase. This competition results in heterogeneity in persister awakening lags. Most persisters repair the oxidative DNA damage, displaying a mutation rate equal to the untreated population. However, the promutagenic factor Mfd increases the mutation rate in a persister subpopulation. Our data provide in-depth insight in the molecular mechanisms underlying persister survival and pinpoints Mfd as an important molecular factor linking persistence to resistance development.

Keywords

Persistence, antibiotic resistance, tolerance, DNA damage repair, transcription-coupled repair

Introduction

Every isogenic bacterial population harbors a subpopulation of non-growing antibiotic-tolerant cells that survive lethal drug exposure, named persisters. Upon antibiotic removal, a persister cell that resumes growth can form a new bacterial population. Persistence is an example of phenotypic bistability leading to the presence of antibiotic-sensitive and -tolerant cells in an isogenic population (1, 2). Evidence for the clinical importance of persister cells is accumulating, as they are held responsible for the recalcitrance of chronic infections (3–6) and they constitute a pool from which resistant mutants emerge (7–10). Indeed, antibiotic-resistant mutants emerged from antibiotic-tolerant cells in *Escherichia coli*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* (8–10). Furthermore, a positive correlation was found between the number of persisters in a bacterial population, and the emergence of resistant mutants in lab and natural *E. coli* strains (7). In this respect, the healthcare system would benefit from the development of anti-persister therapies.

The number of persisters at any given time is determined by a combination of the rates of persister state entry and exit. Several decades of persistence research have resulted in the discovery of different molecular mechanisms triggering persister state entry (1). These mechanisms often include decreased cellular energy levels (11, 12) and/or a block in essential cellular processes such as translation (13, 14) and replication (15). In addition, mechanisms that avoid antibiotic-inflicted DNA damage or antibiotic accumulation in the cell were reported to induce the persister state (16, 17). In stark contrast, only few studies have attempted to investigate mechanisms coordinating persister state exit. This discrepancy mainly results from the technical difficulties in analyzing and quantifying persister awakening, such as the transient phenotype and the small number of persisters in a population. As a result, a systematic understanding of persister state exit is largely lacking.

Recent work has postulated the hypothesis that persister state exit depends on reverting the physiological changes correlated with the induction of the persister state (1). For example, it has been hypothesized that persisters formed by the TacT toxin, which acetylates tRNA, resume growth via Pth-mediated deacetylation (14). Additionally, persisters formed by the pore-forming HokB toxin were found to wake up based on pore disassembly via a combination of DsbC-mediated monomerization and DegQ-mediated peptide degradation, followed by membrane repolarization (18). Furthermore, protein aggregate formation and removal were shown to be correlated with persister state entry and exit (19). Based on these findings, we can infer that other persister formation pathways should also be reverted to initiate persister state exit.

It has long been assumed that persisters have a dormant phenotype which allows them to survive the antibiotic treatment because of target inactivity (20). However, recent work has pointed towards a role for antibiotic-induced damage repair in successful persister awakening. Indeed, both persisters from cultures in stationary or exponential phase were found to acquire DNA-damage after fluoroquinolone treatment (21, 22), contradicting this hypothesis. Later, it was demonstrated that the timing of DNA-damage repair is important for successful persister recovery after antibiotic treatment by DNA-damaging agents. Indeed, if DNA repair was stalled until the initiation of growth-associated processes, persister survival was strongly reduced (23). Furthermore, ploidy was found to enhance persister survival, demonstrating that persisters use homologous recombination (HR) to repair the double-stranded breaks (DSB) (24). Combined, DNA damage repair has proven to be an essential and general phenotype for persister survival following fluoroquinolone treatment, making it a promising target for an anti-persister therapy. However, research to date has not identified any molecular mechanisms that underlie the successful recovery of the persister cell. To address which of these repair proteins are important for successful persister recovery, we systematically tested deletion mutants of DNA-repair genes and examined their expression during recovery following antibiotic treatment.

In this work, five DNA-damage repair genes (*recB*, *recC*, *uvrA*, *uvrB* and *uvrD*) were found to be important for persister survival following fluoroquinolone treatment. RecB and RecC are both involved in double strand break repair via HR (25). The UvrA₂B complex, together with UvrC and UvrD, is involved in nucleotide excision repair (NER) (26). NER repairs a broad variety of bulky, structurally unrelated, DNA damages, such as DNA damage following UV irradiation or hydrogen peroxide treatment (27, 28). Damage recognition is based on either UvrA₂B that scans the entire genome and detects damages that cause a distortion of the double helix (26) or via transcription-coupled repair (TCR), which is based on the detection of DNA lesions that completely stall (29, 30) or pause (31) the RNA polymerase, after which repair is orchestrated by NER. TCR is biased towards actively expressed genes (30, 32). The bacterial cell employs two different TCR pathways. The first one relies on Mfd, which recruits UvrA to the site of the lesion for NER-mediated repair and pushes the RNA polymerase forward, terminating transcription (33, 34). The translocase Mfd is also an evolvability factor and was found to increase mutagenesis (35, 36). A second TCR pathway was found to depend on UvrD, which backtracks the stalled RNA polymerase, after which NER-mediated repair occurs following NusA-mediated recruitment (37). The RNA polymerase is not lost upon backtracking by UvrD, enabling

transcription to resume rapidly upon DNA repair (37). Presence of (p)ppGpp makes the RNA polymerase backtracking-prone (38). After detection of the DNA lesion, UvrC cleaves 5' and 3' from the lesion, after which the helicase UvrD releases the damaged oligonucleotides (26, 39). In addition to its role in NER and TCR, UvrD acts as a helicase in methyl-directed mismatch repair following replication (40) and helps stalled replication forks to restart by removing the Tus protein (41, 42).

In this work, we demonstrate that ofloxacin treatment results in the accumulation of oxidative DNA damage. Transcription re-initiates during recovery in fresh medium, enabling TCR to detect and repair the oxidative DNA damage via NER. Clearing of the RNA polymerase from the site of the lesion occurs via UvrD or Mfd. Single-cell observations of recovering persister cells demonstrate that the UvrD-mediated TCR pathway decreases the awakening lag of the persister cell, while Mfd-mediated TCR increases the awakening lag. Furthermore, Mfd-mediated TCR is responsible for the emergence of a subpopulation of persister cells with a high mutation rate, providing mechanistic insight on the emergence of resistant mutants from a persister population. In addition, phenotypic characteristics of persister cells were compared with sensitive cells, revealing differences in initial elongation rate during the awakening lag and in *mfd* expression levels. Combined, our work is one of the first to explore molecular mechanisms underlying persister awakening dynamics in a wild-type background and provides us with an understanding of the accumulation of mutations in persister cells at the molecular level.

Experimental work

1. Absence of RecB, RecC, UvrA, UvrB and UvrD impedes persister awakening after fluoroquinolone treatment

To define which DNA repair pathways underlie persister cell survival after fluoroquinolone treatment, we hypothesized that absence of a gene important for survival should result in a decreased persister fraction. Therefore, we have first built a comprehensive list of genes coding for proteins that are directly implicated in DNA damage repair (Table S1). Next, the persister fractions of the corresponding deletion mutants were assessed in stationary phase after treatment with the fluoroquinolone antibiotic ofloxacin. Figure 1A demonstrates that absence of *recB*, *recC*, *uvrA*, *uvrB* and *uvrD* results in a strong decrease in the persister fraction following ofloxacin treatment. Time-kill curves were performed to confirm the persister population (Fig. S1A) (43). The lower persister fraction of *recB* and *recC* mutants nicely demonstrates the validity of our hypothesis, as RecB and RecC were recently linked with persister survival following their role in homologous recombination to repair DSB (24). UvrA, UvrB and UvrD are components of the NER pathway, and the molecular mechanisms underlying their role in persistence are unknown (23, 26, 44). We confirmed the decreased survival of these mutants following treatment with the fluoroquinolone moxifloxacin (Fig. S1B).

Persister fractions assessed using plate counts result from the combined effect of rates of persister state entry and exit. To distinguish whether the absence of *uvrA*, *uvrB* and *uvrD* impedes persister formation or awakening, we first assessed the number of metabolically active cells before treatment compared to the number of metabolically active cells after

antibiotic treatment using the metabolic activity sensor redox sensor green (RSG). The green fluorescence is positively correlated with the metabolic activity of the cell. No significant difference of the ratio of RSG+ cells after and before treatment between the $\Delta recB$, $\Delta recC$, $\Delta uvrA$, $\Delta uvrB$ and $\Delta uvrD$ mutants and the wild type was observed (Fig 1B). This demonstrates that an equal fraction of cells is metabolically active after the ofloxacin treatment, suggesting that the deletions do not interfere with direct cell survival following antibiotic treatment. Second, the number of persister cells per metabolically active cell after antibiotic treatment was calculated, as published previously (18). For this, the RSG staining was performed in parallel with CFU counting. Figure 1C clearly shows that absence of $recB$, $recC$, $uvrA$, $uvrB$ and $uvrD$ results in significantly less persisters per metabolically active cells (RSG+) after treatment, indicative of an impaired awakening efficiency. Combined, these data demonstrate that absence of $recB$, $recC$, $uvrA$, $uvrB$ and $uvrD$ disrupts successful persister awakening in ofloxacin-treated cells. As the molecular role of $recBC$ has been studied before in relation to persistence (24, 44), we further focused on the uvr genes.

Figure 1. $\Delta uvrB$, $\Delta uvrD$ and $\Delta recC$ mutants have an impaired awakening. (A) Persister fractions after ofloxacin treatment of stationary phase cells. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. Data are log-transformed. Asterisks indicate significant difference compared to the wild type (*E. coli* BW25513) (**** $P < 0.0001$; ** $P < 0.01$). (B) The number of metabolically active cells after ofloxacin treatment over the number of metabolically active cells before ofloxacin treatment is not significant different in $\Delta recB$, $\Delta recC$,

$\Delta uvrA$, $\Delta uvrB$ and $\Delta uvrD$ compared to the wild type. Metabolic activity is measured using RSG staining. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. (C) After treatment with ofloxacin, the number of regrowing persister cells per metabolically active cell (RSG+) is significantly lower for $\Delta uvrA$, $\Delta uvrB$, $\Delta uvrD$, $\Delta recB$ and $\Delta recC$ compared to the wild type. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. Asterisks indicate significant difference compared to the wild type (*E. coli* BW25513) (**** $P < 0.0001$; ** $P < 0.01$).

2. Single-cell analysis during recovery reveals different awakening kinetics between antibiotic-sensitive and persister cells

To further confirm the role of UvrA, UvrB and UvrD in persister awakening, detailed single-cell awakening kinetics of the corresponding deletion mutants were quantified using time-lapse microscopy (movie S1-S2). In agreement with previous reports (22, 24), the majority of the cells starts elongating during recovery as a response to the ofloxacin treatment (Fig. 2A). However, only persister cells are able to divide multiple times ($n > 4$) and to generate viable progeny.

Elongation of both persister cells and antibiotic-sensitive cells was monitored as a function of time, resulting in detailed quantitative assessment of awakening kinetics for both populations. Because persister cells strongly elongate before cell division following fluoroquinolone treatment, we propose that the total awakening time is characterized by two different phenotypic hallmarks: the awakening lag, defined as the time the cell needs to reach twice its initial size, and the time of cell division.

Single-cell awakening kinetics reveal that the awakening lag of individual persister cells is highly variable, ranging from 75 to 526 minutes in the wild type. Awakening lags of wild-type persisters are normally distributed, pointing towards underlying molecular mechanisms (45). In addition, during the awakening lag, the cellular elongation rate is higher in sensitive cells compared to persister cells (Fig. S2A, S2B and S2C). Absence of UvrA and UvrB does not interfere with the awakening lag (Fig. 2B), but significantly increases the time between the awakening lag and cell division (Fig. 2C). In contrast, absence of UvrD strongly increases the awakening lag. By measuring cell length as a function of time during the awakening lag in persister cells, it is clear that this increase in awakening lag is not caused by a slower cell elongation of the mutant, but a strong delayed initiation of cell elongation compared to the wild type (Fig. S2D). As this effect is specific for $\Delta uvrD$, this might point towards a role for UvrD-mediated RNA polymerase backtracking in the awakening lag of persister cells, which occurs independently of UvrA₂B (37). Absence of UvrD also increases the time of cell division in persister cells (Fig. 2B), although after this extended awakening lag, the time to cell division is comparable to that of the wild type (Fig. 2C). Importantly, this difference in the awakening lag between wild-type persister cells and $\Delta uvrD$ persisters is specific for persister cells, as no significant difference is observed in the antibiotic-sensitive cells (Fig. 2D).

Previous work demonstrated that expression of repair genes is important during the recovery period following ofloxacin treatment (21, 23). Therefore, expression of promoter-gene fusions of the *uvr* genes with *gfp* (46) was monitored as a function of time for the duration of the awakening lag in the wild type following ofloxacin treatment (Fig. 2E). Expression of *uvrA*, *uvrB*

and *uvrD* is induced following ofloxacin treatment in the bulk of the population, although expression of *uvrA* and *uvrD* is more elevated. These data demonstrate that NER is expressed following ofloxacin treatment, and that absence of this repair pathway strongly influences persister awakening kinetics. In a next set of experiments, we investigated the molecular role of NER in persister awakening.

Figure 2. Awakening kinetics of wild-type *E. coli*, $\Delta recB$, $\Delta recC$, $\Delta uvrA$, $\Delta uvrB$ and $\Delta uvrD$. (A) Representative example of wild-type cells after ofloxacin treatment, growing on an agar pad with fresh nutrients. Almost all cells start to elongate after antibiotic treatment, but only persister cells undergo multiple rounds of cell divisions (> 2). The awakening lag is determined as the time point when the cell reaches twofold its initial size. The yellow arrows indicate the cell division planes. (B) Awakening lag and cell division for persister cells. $\Delta uvrD$ persister cells wake up significantly later and have a stalled cell division compared to wild type persister cells. Error bars are represented as mean $\pm$ SEM. Asterisks indicate significant difference (** P < 0.01). (C) The difference between the awakening lag and the time of cell division is significantly longer in absence of UvrA and UvrB. Error bars are represented as mean $\pm$ SEM. Asterisks

indicate significant difference (* $P < 0.05$). (D) The difference in awakening lag is specific for persister cells, as no significant difference is detected in awakening lag of the antibiotic-sensitive cells. Error bars are represented as mean $\pm$ SEM. (E) Following ofloxacin treatment, wild-type strains containing *pUvrA-gfp*, *pUvrB-gfp* and *pUvrD-gfp* were washed and diluted in LB, after which fluorescence intensity was assessed after 0, 60, 120, 180 and 240 minutes of incubation in fresh medium using flow cytometry. As a control, fluorescence intensities of untreated strains were taken. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM.

3. Persister cells use NER to repair oxidative DNA-damage

Fluoroquinolones such as ofloxacin result in the formation of DSB, which are repaired via HR (21, 24). The molecular role for UvrA, UvrB and UvrD in persister survival after ofloxacin treatment is therefore likely indirect, as NER does not contribute to DSB repair. NER is involved in the repair of a broad variety of other DNA lesions which are detected either directly via the UvrA₂B complex, or indirectly via transcription-coupled repair (TCR) (26, 27, 29, 30, 37). One of the well-known damages directly detected and repaired by NER are pyrimidine dimers which are formed following UV radiation. These pyrimidine dimers are, however, not formed following ofloxacin treatment (Fig. S3).

Several studies demonstrate that antibiotics induce the formation of reactive oxygen species (ROS) (47–49). Accumulation of ROS induces several types of DNA lesions, which can induce cell death if left unrepaired or when repair is unsuccessful, for example by a defective NER machinery (48, 50–52). We therefore first investigated whether ROS are induced in our set-up. ROS production after ofloxacin treatment was confirmed using the non-fluorescent precursor H₂DCFDA (2,7-dichlorodihydrofluorescein) (Fig. 3A), which can be oxidized to the green-fluorescent molecule H₂DCF. Accumulation of ROS can result in DNA damage, for example via the incorporation of oxidized nucleotides (47, 52), with 8-oxoguanine as the major product (53). To investigate whether the observed increase in ROS following ofloxacin treatment results in oxidative DNA lesions, the formation of 8-oxoguanine was assessed after ofloxacin treatment and during recovery. Therefore, antibiotic-treated cells were fixed after 0, 1 and 3h during recovery in fresh medium, which corresponds to the awakening lag in the persister cells. Untreated cells were used as a control. Following fixation, anti-8-oxoguanine antibodies were applied and the cells were visualized under the microscope, after which the fluorescence intensity relative to the control cells was calculated (Fig. 3B). Here, an increase of 8-oxoguanine nucleotides is observed after 0, 1 and 3h recovery in fresh nutrients, confirming that ofloxacin treatment results in the accumulation of an oxidized nucleotide pool, leading to oxidative DNA damage upon incorporation.

In a next step, we assessed the essential role of NER in the repair of oxidative DNA damage. Therefore, a persister assay was performed in anaerobic conditions to prevent the formation of ROS during ofloxacin treatment and persisters were quantified in different NER mutant backgrounds (Fig. 3C). To confirm the accumulation of DSB in anaerobic conditions, strains devoid of RecB and RecC, mediating DSB repair via HR, were taken as a control. Under anaerobic conditions, a significant decreased persister fraction is observed for $\Delta recB$ and $\Delta recC$, but not for $\Delta uvrA$, $\Delta uvrB$ or $\Delta uvrD$, pointing towards a role for NER in oxidative DNA

damage-repair during persister recovery, and confirming that NER does not have a function in DSB repair.

Figure 3. Repair of oxidative DNA damage influences persister survival. (A) ROS production after ofloxacin treatment was confirmed using the non-fluorescent precursor H₂DCFDA, which emits green light upon oxidation to H₂DCF. Green fluorescence was measured using flow cytometry, and the mean fluorescence of the population was calculated. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. Asterisks indicate significant difference (** $P < 0.01$). (B) Ofloxacin treatment results in the formation of 8-oxoguanine. Primary anti-8-oxoguanine antibodies were used, after which an Alexa Fluor secondary antibody, linked with a fluorescent protein, was used to quantify 8-oxoguanine formation. The mean fluorescence of the cell was measured using fluorescent microscopy. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. Asterisks indicate significant difference with the untreated control condition (**** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$). (C) Samples were grown and treated with ofloxacin in strict anaerobic conditions. Data are log-transformed. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. Asterisks indicate significant difference compared to the wild type (*** $P < 0.001$; ** $P < 0.01$).

4. TCR underlies the variation in awakening lag in persister cells

In *E. coli*, different mechanisms are at play to detect and repair different types of oxidative DNA damage (27, 54). Previous experiments demonstrate that persister survival following ofloxacin treatment is based on NER-mediated repair of oxidative DNA damage. NER-mediated repair can occur directly, if the UvrA₂B complex identifies the lesion, or indirectly following detection by transcription-coupled repair (TCR), where a stalled or impeded RNA polymerase is recognized and displaced by Mfd (31, 55) or moved backwards by UvrD (37). Importantly,

single-cell time lapse microscopy (Fig. 2B) demonstrates that only the absence of UvrD strongly increases the awakening lag of persister cells, but not the absence of UvrA and UvrB. This implies that the role of UvrD in TCR, rather than its role in NER, governs the awakening lag. This might suggest that changes in TCR functioning will alter awakening kinetics, rather than antibiotic survival. To confirm this hypothesis, persister assays were performed on several deletion mutants influencing TCR: $\Delta mfd\Delta uvrD$, Δmfd and $\Delta greA$. In absence of UvrD and Mfd, the cell is devoid of a dedicated RNA polymerase displacement factor, ruling out TCR. Furthermore, GreA is an anti-backtracking factor (37, 56), of which deletion results in a backtracking-prone phenotype (37). No significant difference in survival of these mutants was observed compared to the wild type (Fig. 4A). These data demonstrate that oxidative DNA damage in persisters following ofloxacin treatment is not necessarily lethal. Of note, $\Delta mfd\Delta uvrD$ has an increased persister fraction compared to $\Delta uvrD$, indicating that in absence of TCR, UvrA₂B is not able to recognize the damage. In these conditions, repair is not initiated and survival is not impaired by the absence of UvrD.

Next, single-cell awakening lags were determined for $\Delta mfd\Delta uvrD$, Δmfd and $\Delta greA$ (movie S3-S4). The $\Delta mfd\Delta uvrD$ mutant has a strongly increased awakening lag (Fig. 4B), which is even more increased compared to $\Delta uvrD$ persisters (Fig. 2A), presumably caused by the absence of a dedicated RNA displacement factor. Strikingly, Δmfd -persister cells have a significantly shorter awakening lag compared to wild-type persister cells (Fig. 4B), which can be explained by the overall backtracking of the RNA polymerase in this mutant, resulting in the fast resumption of transcription. Although not significant ($p = 0.099$), a downward trend, similar to Δmfd , is observed for $\Delta greA$ -persisters. This is in agreement with the backtracking-prone phenotype of $\Delta greA$ (37), of which the phenotype is weaker compared to complete absence of Mfd. Absence of both Mfd and GreA results in significantly less variation in awakening lag compared to wild-type cells (Levene's test, $p = 0.00016$ and $p = 0.013$, respectively). Importantly, no significant difference in awakening lag is observed in the sensitive cells for Δmfd and $\Delta greA$ (Fig. S4A). Interestingly, we also observe a significant difference in awakening lag in antibiotic sensitive cells when we compare wild-type and $\Delta mfd\Delta uvrD$ backgrounds. However, the difference with the wild-type is strikingly higher in persister cells, demonstrating a persister-specific effect.

These data suggest that stalling of the RNA polymerase by oxidative DNA damage influences the awakening lag via an interplay between the two TCR pathways, orchestrated by UvrD and Mfd. In absence of Mfd, a stalled RNA polymerase will be rescued by UvrD-mediated backtracking. The damage will be repaired and transcription can proceed swiftly as UvrD will backtrack the RNA polymerase (37), which will result in the observed short awakening lag and the lower variation (Fig. 4B). In absence of UvrD, Mfd will displace the RNA polymerase and transcription has to restart, resulting in the observed strongly increased awakening lag (Fig. 2B). To further validate the competitive interaction between UvrD- and Mfd-mediated TCR and their influence on the awakening lag of the persister cells, expression of *uvrD* and *mfd* was monitored in persister cells during recovery using time-lapse microscopy. A positive correlation is observed between the timing of *uvrD* expression and the awakening lag at single-cell level (Fig. 4C), suggesting that early expression of *uvrD* is correlated with a short awakening lag. Furthermore, a negative correlation is observed between the timing of *mfd* expression and the

awakening lag, suggesting that early *mfd* expression during persister recovery is correlated with a long awakening lag (Fig. 4D). Strikingly, persister cells have an increased expression of *mfd*, but not *uvrD*, following ofloxacin treatment compared to antibiotic-sensitive cells (Fig. S4B, S4C).

Previous data demonstrate that TCR alters the awakening lag of the persister cell in response to oxidative DNA damage. To further corroborate the influence of oxidative DNA damage on the awakening lag of persister cells, ofloxacin treatment and recovery of wild-type cells were performed in combination with the hydroxyl radical scavenger thiourea (TU) (57). Hydroxyl radicals are the main contributor to the formation of 8-oxoguanine. Treatment with TU results in a significantly decreased awakening lag (Fig. 4E), but again, did not alter persister survival (Fig. S4D). Consistent with previous data, a significantly decreased variation in awakening lag is observed in wild-type persister cells treated with TU (Levene's test, $p = 0.0047$).

Combined, these data demonstrate that oxidative DNA damage does not necessarily cause cell death in a wild-type background, but initiates TCR-mediated NER, resulting in heterogeneity of the awakening lag in persister cells. If repair fails, for example when the NER machinery is defective, survival is impaired.

Figure 4. Variation in awakening lags in persister cells is caused by TCR. (A) Persister fractions after ofloxacin treatment of $\Delta mfd\Delta uvrD$, Δmfd and $\Delta greA$. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. Data are log-transformed. Asterisks indicate significant difference compared to the wild type (*E. coli* BW25513) (**** $P < 0.0001$). (B) Single-cell awakening kinetics of persister cells following recovery on LB agar pads after ofloxacin treatment. $\Delta mfd\Delta uvrD$ persisters have a significantly increased awakening lag compared to wild-type persister cells. Δmfd persisters have a significantly shorter awakening lag compared to wild-type persister cells. Error bars are represented as mean $\pm$ sd. Asterisks indicate statistical significance (**** $P < 0.0001$, * $P < 0.05$) compared to the wild type. A downward

trend for $\Delta greA$ persisters is observed, but it is not significant ($p = 0.099$). The variation of the awakening lags of Δmfd - and $\Delta greA$ persisters differs significantly compared to the wild type (Levene's test, $p = 0.00016$ and $p = 0.013$, respectively). (C) Wild-type strains containing pUvrD-gfp were treated with ofloxacin, after which their recovery was followed at the single-cell level using time lapse microscopy. Fluorescence intensity and cell length were followed in time for 15 persister cells. The time of *uvrD* expression was significantly correlated with the awakening lag ($p = 0.0186$). Correlation was calculated using Spearman ($R = 0.6071$). (D) Wild-type strains containing pMfd-gfp were treated with ofloxacin, after which their recovery was followed at the single-cell level using time lapse microscopy. Fluorescence intensity and cell length were followed in time for 17 persister cells. The time of *mfd* expression was significantly correlated with the awakening lag ($p = 0.0158$). Correlation was calculated using Spearman ($R = -0.5449$). (E) Single-cell awakening kinetics of wild-type persister cells with and without TU treatment following recovery on LB agar pads after ofloxacin treatment. Cells treated with TU have a significantly shorter awakening lag compared to the untreated control. Error bars are represented as mean $\pm$ sd. Asterisks indicate statistical significance (* $P < 0.05$).

5. Mfd-mediated TCR plays a role in the emergence of resistant mutants.

If left unrepaired, oxidative DNA damage such as 8-oxoguanine can be mutagenic (52). Previous work has established that persisters form a pool from which resistant mutants emerge (7–9). Furthermore, Mfd is an evolvability factor that increases mutation rates and has been linked with the emergence of antibiotic resistance (35, 36). Therefore, to address the role of oxidative stress and oxidative DNA damage repair on the accumulation of mutations in persister cells, a fluctuation assay was performed (58). Here, the distribution of the emergence of mutants in multiple parallel cultures is determined, after which the mutation rate is inferred from this distribution of mutants (58, 59). The mutation rates in wild-type, Δmfd - or $\Delta uvrD$ -backgrounds did not differ between untreated and treated populations (Fig. 5A), meaning that the overall probability of a mutation occurring per cell division is not different between untreated and ofloxacin-treated cells (59, 60). However, an increased mutation rate is observed in $\Delta uvrD$, both in untreated and treated cultures, compared to the wild-type. An increased mutation rate in $\Delta uvrD$ was observed before (61) and was shown to be the result of the role of UvrD in methyl-directed mismatch repair (MMR), which corrects replication errors (40, 62).

Importantly, a previous report demonstrated that a population treated with fluoroquinolones has a higher mutation frequency (63). The mutation frequency is determined as the proportion of mutants in a culture and is strongly biased if a mutational event occurs early in exponential phase (59). We hypothesized that this discrepancy was the result of a higher mutation rate occurring sporadically in a very limited number of persister cells. Therefore, different bottlenecks of populations were tested. Populations were treated with antibiotics or left untreated, and subsequently 10^4 or 10^6 cells were transferred to fresh medium. The mutation frequency was assessed after overnight incubation (Fig. 5B). No significant increase in mutation frequency is found upon recovering 10^4 cells, which is the same population size as that used in the fluctuation assay in Fig. 5A. However, when 10^6 cells are taken, an increased number of mutants per generation upon ofloxacin treatment in the wild type is observed compared to the untreated control (Fig. 5B). Given the promutagenic role of Mfd and the fact that absence of Mfd decreases the formation of resistant mutants (35), we hypothesized that Mfd might be

responsible for these sporadic mutational bursts in persister cells. In absence of Mfd, no significant difference in the mutation frequency per population bottleneck is observed ($p = 0.22$), indicating that Mfd is indeed responsible for bursts in mutational events in persister cells following ofloxacin treatment (Fig. 5C).

According to these data, we can infer that most persisters do not have an altered mutation rate. However, a small subpopulation of persister cells have an elevated mutation rate and are more likely to form resistant mutants, triggered by Mfd.

Fig. 5. The persister population contains different subpopulations with different mutation rates. (A) No significant difference is observed in mutation rates, inferred by performing a fluctuation assay, between untreated (control) and ofloxacin-treated populations. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. (B) Taking an increased bottleneck population (10^6) following ofloxacin survival significantly increases the mutation frequency per generation in the wild type. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM. (C) Taking an increased bottleneck population (10^6) following ofloxacin survival significantly does not increase the mutation frequency per generation in absence of Mfd. Values result from $n \geq 3$ biological repeats and are represented as mean $\pm$ SEM.

Discussion

To date, persistence research has mainly focused on mechanisms involved in the induction of the persister state. From a medical point of view, targeting persister awakening is much more interesting than targeting persister formation as a bacterial infection likely already accommodates persister cells at the moment of treatment. As persisters accumulate damage during fluoroquinolone treatment, the ensuing recovery response is a promising target for an anti-persister therapy. Therefore, detailed insights in the molecular mechanisms underlying damage repair during persister awakening are of critical importance.

In this work, we deepened the role of DNA damage repair in persister survival after ofloxacin treatment. Fluoroquinolones such as ofloxacin result in the formation of DSB by blocking DNA-gyrase functioning. The formation of these DSB in persister cells was reported previously (21) and it was demonstrated that persister cells containing two chromosomes are more likely to survive fluoroquinolone treatment, resulting from the ability to perform HR (24). This is in agreement with our observation that the presence of RecB and RecC is important for successful persister survival, as in *E. coli* HR is predominantly orchestrated via the RecBCD pathway (64).

In addition to HR, our results clearly point towards a prominent role for NER in persister survival by oxidative DNA damage repair. Our work and others have demonstrated that antibiotics induce the formation of ROS (47, 48, 50, 51). Whether or not this accumulation results in cell death is under debate (49, 65). Our results demonstrate that the oxidative DNA damage following ofloxacin treatment does not induce cell death directly in a wild-type background, but can cause cell death if repair is corrupted, which is in line with previous reports (52, 66). Although the repair of oxidative DNA damage is often attributed to base-excision repair mechanisms (BER) (52), recent work has demonstrated that NER is important for survival of oxidative stress when the level of damage is high (27). Our results demonstrate that in *E. coli* persisters, the NER pathway repairs oxidative DNA damage, as absence of UvrA, UvrB and UvrD significantly lowers persister survival during recovery in aerobic conditions. Of note, UvrD was linked with decreased antibiotic survival in *E. coli* MG1655 (23, 44), suggesting a general mechanism.

Our results show that detection of the oxidative DNA damage occurs via TCR. Although under debate (29, 55), oxidative DNA damage can stall or pause RNA polymerase, which is recognized and displaced by Mfd (31, 55) or moved backwards by UvrD (37) to expose the site of the lesion and enable repair. In both cases, this repair is orchestrated via NER. While transcription can swiftly proceed after repair if the RNA polymerase is moved backwards by UvrD, transcription terminates following Mfd-mediated forward movement of the RNA polymerase (33, 37, 56). Hence, the molecular mechanisms underlying movement of the RNA polymerase dramatically change transcription dynamics, and as a result, the awakening lag of the persister cells. UvrD-mediated backtracking results in a short awakening lag, while Mfd-mediated displacement of the RNA polymerase results in a long awakening lag of the persister cells. Importantly, the role of UvrD is twofold: while our data demonstrate that its role in NER-mediated repair as a helicase will influence persister survival, its role in TCR influences the awakening lag. At the population level, these differences in transcription dynamics and competition between these systems will result in heterogeneous persister awakening, as was observed before (22, 24). We can speculate that this phenotypic variability aids in survival of the persister population upon antibiotic encounter, as it diverges the timing that persister cells become antibiotic-sensitive again (67).

Some of the oxidative DNA damage, such as 8-oxoguanine, is mutagenic upon incorporation in the DNA. Previous work has suggested that the accumulation of ROS following antibiotic treatment plays a role in resistance development (9, 68). In addition, increased oxidative stress in biofilms increases mutation frequencies in *Staphylococcus aureus* (69). Our results demonstrate that following antibiotic treatment, the majority of the persister cells repair the oxidative DNA damage, and have a mutation rate equal to the untreated population. However, a small subpopulation of persister cells has a higher mutation rate, triggered by Mfd, resulting in the emergence of resistant mutants (Figure 6). Indeed, in addition to its role in repairing DNA damage, Mfd was found to play a role in the evolution of antimicrobial resistance in *Salmonella typhimurium*, *Bacillus subtilis*, *M. tuberculosis* and *Campylobacter jejuni* (35, 70) and in stationary-phase mutagenesis in *Pseudomonas putida* and *Bacillus subtilis* (61, 71). This promutagenic function presumably works via the formation of R-loops, inherent to transcription, where one strand of DNA is annealed to a complementary RNA strand instead of

its complementary DNA strand (36). These R-loops are precursors for both DSB and double-stranded ends (DSE), which are mutagenic both during replication and in starvation-stressed cells (72). In stationary phase, TCR predominantly occurs via UvrD-mediated backtracking (37, 38). Hence, these jackpot events are rare under these conditions, however, they have a high biological relevance if the number of residing persisters at an infection site following antibiotic treatment is high. Importantly, subpopulations with different mutation rates were observed before and it was postulated to be a bet-hedging strategy (67, 68, 73). Our work links extended awakening lags with a higher mutation rate (Figure 6), shifting the balance between genome integrity and plasticity in time, which might be a strategy of the persister population to preserve genome integrity of the population if mutagenesis is not needed (74).

Persister cells and sensitive cells were found to have three major behavioral differences following antibiotic treatment. First, persister cells have a significantly decreased rate of elongation compared to antibiotic-sensitive cells. Second, our data demonstrate that the influence of TCR on the awakening lag is specific for persister cells. However, the observation that sensitive cells also elongate strongly suggests that transcription is initiated as well. Third, persister cells have a significantly increased *mfd* expression compared to sensitive cells immediately after ofloxacin treatment. In another study, increased expression of *mfd* was also observed following ciprofloxacin treatment in *C. jejuni* (70). Presumably, these differences will change awakening kinetics and alter transcription dynamics, resulting in the observed difference between persister cells and sensitive cells. It is tempting to speculate that alteration of transcription speed increases the chance of DSB repair by halting growth resumption, thereby increasing the chance of survival (23, 75).

The findings reported here shed new light on the role of NER in successful persister recovery following fluoroquinolone treatment and demonstrate that TCR dictates the heterogeneity of awakening lag of persister cells. In addition, we reveal the presence of a subpopulation of persisters with a higher mutation rate, driven by the promutagenic factor Mfd (Figure 6). Finally, an increased awakening lag seems correlated with a higher mutation rate. This way, the chance of survival of the bacterial population is increased, while genome integrity is maintained.

Figure 6. Model of the influence of TCR on the awakening lag of persister cells following fluoroquinolone treatment. Upon stalling of the RNA polymerase by oxidative DNA damage, TCR is initiated to remove the RNA polymerase from the site of the lesion (green square), enabling NER to repair the damage. UvrD-mediated movement of the RNA polymerase results in direct proceeding of transcription, resulting in a short awakening lag of the persister cell. Mfd-mediated displacement of the RNA polymerase terminates transcription, increasing the awakening lag of the persister cell. In addition, the promutagenic factor Mfd will result in jackpot events in a small fraction of the persister cells, leading to antibiotic resistance.

Materials and methods

Bacterial strains, growth conditions and plasmids.

The *E. coli* strain BW25113 was used for all experiments (76). Bacteria were grown in lysogeny broth (LB) at 37°C under orbital shaking (200 rpm). When needed, kanamycin was added (40 µg mL⁻¹ for genomic deletion mutants and 20 µg mL⁻¹ for the promoter fusions) to the culture.

Construction of pUvrB was performed by PCR amplification of the intergenic region (500 bp) preceding the start codon of the *uvrB* gene using primers ACT GCT CGA GCC ATT CTG TAT TTG GTT AAA and CAC CGG ATC CGA GTC GCT ACC TGA AGG AGT. Construction of pRecC was performed by PCR amplification of the promoter region of *recC* using primers ACT GCT CGA GAT GTC AGC TTC CCT GAA GAA and CAC CGG ATC CAG CGG CTC CTG ACT ACT GAC. The corresponding PCR products were digested with XhoI and BamHI and cloned in PUA66 (46). pRecB was constructed using Gibson assembly. The promoter region of *recB* was amplified using TTC TTA AAT CTA GAG GAT CCT CAC GGA CCT CAT AAT CAA CTT AAT TTT CTGT and CTT TCG TCT TCA CCT CGA GAG TGC TGT ATA AAA ATT GCG CAA TCT. The PUA66 plasmid was linearized using XhoI and BamHI. Gibson assembly was performed using the Gibson assembly master mix[®] purchased from New England Biolabs. pUvrA and pUvrD were purified from the library constructed in (46) and transformed to BW25113.

Persistence assay

Cells were inoculated in 5 mL LB medium and incubated for 24 h at 37°C under orbital shaking. This preculture was diluted 100-fold in 5 mL LB medium and again incubated for 16 h. Then, 990 µL of the culture was treated with 10 µL ofloxacin with a final concentration of 5 µg ml⁻¹ or with 10 µL moxifloxacin with a final concentration of 2.4 µg ml⁻¹. As a control, 990 µL of the culture was treated with 10 µL sterile water. Cultures were treated for 5 h under shaking conditions at 37°C. Scavenging of the hydroxyl radicals was performed by adding 150 µM thiourea during the ofloxacin treatment. The antibiotic treated samples were washed two fold in 10 mM MgSO₄ to remove the antibiotics, after which all samples were serially diluted in 10 mM MgSO₄ and plated out on LB agar plates. The number of colony forming units (CFU) was determined after 48 h of incubation at 37°C. The persister fraction is determined as the log₁₀ transformation of the number of cells after treatment over the number of cells before treatment.

The persister assay in anaerobic conditions was performed in a Whitley DG250 anaerobic workstation (flushed with 80% N₂, 10% CO₂ and 10% H₂). Prior to usage, all media were incubated for 24 h in the anaerobic workstation. Statistical comparisons were based on a one-way ANOVA compared to the wild type with a Dunnett's *post hoc* test for multiple comparison.

Determination of persister cells in the metabolically active fraction

To determine the number of persister cells in the metabolically active fraction, a persistence assay was performed as described above. Parallel with plating out the samples, the samples were treated with redox sensor green, purchased from Invitrogen (USA). Therefore, the control cultures were diluted 10-fold and 2 µL of this dilution was dissolved in 198 µL PBS containing RSG, according to the manufacturer's specifications. 2 µL of the antibiotic-treated samples were directly diluted in 198 µL PBS with RSG. After 10 min incubation at room temperature, the number of metabolically active cells per mL were counted using a cytoFLEX S (Beckman Coulter, USA). Data were analyzed using CytExpert (Beckman Coulter, USA). The number of metabolically active cells after treatment was divided over the number of metabolically active cells before treatment. In addition, the number of regrowing cells on plate was divided over the number of metabolically active cells measured using the cytoFLEX S, and the data were log-transformed. Data were analyzed using a one-way ANOVA test compared to the wild type with a Dunnett's *post hoc* analysis for multiple comparison.

Determination of gene expression during recovery after antibiotic treatment using flow cytometry

Cells were incubated and treated with antibiotics as described for the persistence assay. After ofloxacin treatment, 500 µL of the antibiotic-treated samples were washed by centrifuging at 6000 rpm for 5 min, after which the pellet was diluted in 500 µL PBS. 10 µL of this resuspension was pipetted in an Eppendorf tube containing 990 µL LB and 20 µg ml⁻¹ kanamycin. 10 µL of the control culture was diluted directly in an Eppendorf tube containing 990 µL LB and 20 µg ml⁻¹ kanamycin. Samples were taken at dedicated time points and green fluorescence (488 nm) was measured in a CytoFLEX S benchtop flow cytometer. Data were analyzed using CytExpert, determining the mean green fluorescence intensity of the bacterial population.

Single-cell analysis of persister cells and antibiotic-sensitive cells using microscopy

Cells were incubated and treated with antibiotics as described for the persistence assay. After ofloxacin ($5 \mu\text{g mL}^{-1}$) treatment, cells were washed twice by centrifuging at 6000 rpm for 5 min and resuspending the cells in preheated (37°C) LB medium. After washing, $2 \mu\text{L}$ of the culture was spotted on LB agar pads. For the treatment with thiourea, $150 \mu\text{M}$ was added to $990 \mu\text{L}$ culture in combination with the ofloxacin treatment, as described before. After 5 h incubation, cells were washed twice, as described before. $150 \mu\text{M}$ thiourea was added to the agar pad. Phase contrast images and, if needed, fluorescence images were taken every 15 min using a Nikon Eclipse Ti-E inverted microscope with a CFI Plan Apochromat 100X objective with a numerical aperture of 1.45. Green fluorescent images were taken using an exposure time of 20 ms and an analog gain of 6.4. Images were analyzed using the NIS Elements Analysis 4.3 software (Nikon, Japan). The cell length was followed in time. When the cell reaches twofold its initial size, this time point was set as the awakening lag. When a cell division plane was visible, this time point was set as the time of cell division.

Normal distribution of the data was assessed using the Shapiro-Wilk test. If the data were not normally distributed, Box Cox transformation was applied to normalize the data prior to statistical testing. For Fig. 2B, data were analyzed using a one-way ANOVA test compared to the wild type with a Dunnett's *post hoc* analysis for multiple comparison. For Fig. 2C, data were analyzed using a Kruskal-Wallis test compared to the wild type with a Dunnett's *post hoc* analysis for multiple comparison. For Fig. 2D, data were analyzed using a one-way ANOVA test compared to the wild type with a Dunnett's *post hoc* analysis for multiple comparison, following Box-Cox transformation ($\lambda = -0.6$) of the awakening lag to normalize the data. For the awakening lag in Fig. 4B, statistical significance was calculated by normalizing the awakening lags using Box Cox ($\lambda = -0.1$). Data were analyzed using a one-way ANOVA. For Fig. 4E, statistical significance was calculated by using a one-sided Welch's t-test. Differences in variation were assessed using a Levene's test. For Fig. S4A, statistical significance was calculated by normalizing the awakening lags using Box Cox ($\lambda = -0.8$). Data were analyzed using a one-way ANOVA compared to the wild type with a Dunnett's *post hoc* analysis for multiple comparison.

An exponential curve ($y=y_0e^{kx}$) was plotted on the cell length in time for the awakening lag using Graphpad Prism, inferring the initial cellular elongation rate (k). Statistical significance was assessed using an unpaired two-sided t-test.

The fluorescence at the start of the experiment was set as the initial fluorescence. Statistical significance was assessed using a unpaired two-sided t-test. The timing of fluorescent expression was determined by plotting a one-phase decay ($Y=(Y_0 - \text{Plateau}) \cdot \exp(-K \cdot X) + \text{Plateau}$) on the fluorescence intensity over time (77), with a 95% confidence interval. K was constrained at 0.01264, based on the average K of 10 randomly selected bleaching curves. If the fluorescence intensity at a given time point exceeded the 95% confidence interval, this time point was set as the time point of expression. The timing of expression was correlated with the awakening lag of the persister cells using Spearman.

Luria Delbruck fluctuation assays

Cells were incubated and treated with antibiotics as described for the persistence assay. Next, an inoculum mixture was prepared in LB containing 10^4 cells. This inoculum mixture was plated

out to verify the initial cell number. For every strain, 16 wells of a 96-well plate were filled with 200 μL of the inoculum mixture. Next, the strains were incubated for 24 h at 37°C and 200 rpm. After overnight growth, 4 of the 16 wells were diluted in MgSO_4 and plated out on LB plates to quantify the number of cells in the culture. The content of the 12 other wells was plated on rifampicin plates (100 $\mu\text{g mL}^{-1}$). Rifampicin plates were stored and incubated dark conditions. All plates were incubated at 37°C and counted after 2 days.

The total cell number (N_{Cells}) was calculated by taking the average of CFU mL^{-1} of the 4 non-selective plates. Mutation rates were calculated using bzrates (<http://www.lcqb.upmc.fr/bzrates>) and $\log_{10}$ -transformed. Statistical analysis was performed using an ANOVA with Šidák correction, where the treated population was compared to the untreated population.

Population mutation rates

Cells were incubated and treated with antibiotics as described for the persistence assay. Next, 10^6 or 10^4 cells were transferred to tubes containing 5 mL LB. The original culture was plated out to assess initial cell number. After 24 h incubation, a tenfold dilution series was made in MgSO_4 and the culture was plated out on an LB plate, assessing the total number of cells. 200 μL of the culture was plated out on rifampicin plates (100 $\mu\text{g mL}^{-1}$). Rifampicin plates were stored and incubated in dark conditions. All plates were incubated at 37°C and counted after two days. The number of mutants was divided over the total number of cells. To compensate for the difference in the number of cell divisions that can occur between the different population sizes, the mutation rate of the cultures was multiplied by $\log(100)/\log(2)$ for the 10^6 population. Data were analyzed using ANOVA with Šidák correction, where the treated population was compared to the untreated population.

Measuring formation of thymine dimers

A persistence assay was performed as described before. Samples were taken immediately after antibiotic treatment, and a non-treated sample was taken as a control. To assess the influence of a recovery phase on the formation of thymine dimers, 800 μL of an untreated and antibiotic-treated sample were taken and centrifuged for 5 min at 6000 rpm, after which the pellet was dissolved in 800 μL fresh LB medium. This was done twice to assure the complete removal of ofloxacin. Samples were incubated for 30 min at 37 °C, after which they were fixated. As a positive control, 1mL of stationary phase cells were pipetted in an empty petri dish and spread out. The petri dish was kept for 30 seconds on a transilluminator, after which the cells were collected in an Eppendorf tube.

Fixation and staining of the cells was performed as described before (78), with some slight adaptations. 800 μL of the samples were centrifuged 6000 x g for 5 min, after which the pellet was dissolved ice-cold 3:1 methanol-acetic acid mixture and stored at -20°C for 5 min. After fixation, cells were centrifuged at 12000 x g for 5 min, and the pellet was washed with 70 % ethanol and stored at 4° for 45 min. Cells were centrifuged at 12000 x g for 5 min, after which the pellet was dissolved in 70% ethanol containing 0.1 M NaOH. Following centrifugation (12000 x g, 5 min), the pellet was washed twice with PBST (PBS containing 0.05 % Tween 20).

The cells were incubated with anti-thymine dimer antibody (diluted 1:2000) (RRID:AB_261597), purchased from Sigma, for 18 h at room temperature in PBST containing 1 % bovine serum albumin with orbital shaking (80 rpm). After overnight incubation, cells were washed twice with PBST. Cells were incubated with Alexa Fluor® 488 goat anti-mouse IgG1 antibodies (diluted 1:10000) (RRID:AB_2535764) for 3 h at room temperature in PBST under orbital shaking (80 rpm). Secondary antibodies were purchased from ThermoFisher. Localization of the fluorescence was determined using a Nikon Eclipse Ti-E inverted microscope with a CFI Plan Apochromat 100X objective with a numerical aperture of 1.45 by spotting 2 µL of the culture on agarose agar pads (2 %). This confirmed that upon UV exposure, fluorescent foci were formed in the cell. Green fluorescent images were taken using an exposure time of 20 ms and an analog gain of 6.4. Images were analyzed using the NIS Elements Analysis 4.3 software (Nikon) by measuring the fluorescence intensity of the whole cell. For assessing the fluorescence of the population, cells were diluted 100-fold in PBS and green fluorescence (488 nm) and were measured using a cytoFLEX S (Beckman Coulter, USA). Data were analyzed using CytExpert (Beckman Coulter, USA), determining the mean green fluorescence intensity of the bacterial population. Statistical comparisons were based on ANOVA, compared to the untreated bacterial culture, with a Dunnett's *post hoc* test for multiple comparison.

Measuring formation of 8-oxoguanine

A persistence assay was performed as described earlier. Immediately after antibiotic treatment, 400 µL of the culture was centrifuged (6000 rpm, 5 min) and washed with fresh LB medium. Next, the culture was diluted 100-fold in an Eppendorf tube containing 1200 µL LB. Samples (200 µL) were taken immediately, or after 1, 2 and 3h incubation at 37°C.

Fixation and staining of the cells was performed as described for the detection of thymine dimers, with some optimization for the used antibodies. Ice-cold 3:1 methanol-acetic acid mixture (200 µL) was added to the samples, after which the samples were incubated on ice for 5 min. After this fixation step, samples were centrifuged at 12000 x g for 5 min, after which the pellet was dissolved in 70 % ethanol and stored at 4°C for 10 min. Cells were centrifuged at 12000 x g for 5 min, after which the pellet was dissolved in 70% ethanol containing 0.1 M NaOH. Following centrifugation (12000 x g, 5 min), the pellet was washed twice with PBST (PBS containing 0.05 % Tween 20).

The cells were incubated with anti-oxoguanine antibodies (diluted 1:2000) (RRID:AB_11210698), for 16 h at room temperature in PBST containing 2 % bovine serum albumin under orbital shaking (80 rpm). Following incubation, samples were washed twice with PBST, after which they were incubated for 2 h with Alexa Fluor® 488 goat anti-mouse IgM antibodies (1:10000) (RRID:AB_2801490) dissolved in PBST containing 2 % bovine serum albumin. After incubation, samples were washed and resuspended in PBST. Cells were visualized using a Nikon Eclipse Ti-E inverted microscope with a CFI Plan Apochromat 100X objective with a numerical aperture of 1.45 by spotting 2 µL of the culture on agarose agar pads (2 %). Fluorescence was distributed over the cell, as expected. Green fluorescent images were taken using an exposure time of 20 ms and an analog gain of 6.4. Images were analyzed using the NIS Elements Analysis 4.3 software (Nikon) by measuring the mean fluorescence intensity of the cell.

Fluorescence intensities between biological repeats varied resulting from small unavoidable changes in the protocol, such as room temperature. Therefore, for every biological repeat, fluorescence intensities were divided over the average fluorescence intensity of the control sample. Data were analyzed using a one-way ANOVA test compared to the control condition (no ofloxacin treatment) with a Dunnett's *post hoc* analysis for multiple comparison.

Measuring formation of ROS

A persistence assay was performed as described above. 500 µL of each culture was washed and diluted in PBS containing 10 µM H₂DCFDA (Sigma-Aldrich). Tubes were incubated at 30 min in the dark at room temperature. Fluorescence was measured using a cytoFLEX S (Beckman Coulter, USA). Data were analyzed using CytExpert (Beckman Coulter, USA), determining the mean green fluorescence intensity of the bacterial population. Statistical analysis was performed using a two-sided t-test.

Author contributions

Conceptualization: D.W. and J.M., Methodology: D.W., Validation: D.W. Investigation: D.W., C.F. and P.M., Writing – original draft: D.W., Writing – review & editing: D.W. and J.M., Visualization: D.W., Supervision: J.M.

Acknowledgements

We thank Prof. Chris Michiels for the use of the anaerobic workstation. The work was supported by grants from the Research Foundation Flanders (FWO; G0B0420N, G055517N), KU Leuven (C16/17/006) and VIB. D.W. received a postdoctoral mandate from the FWO (1204621N).

Declaration of interest

The authors declare no competing interests.

Citing literature

1. Wilmaerts D, Windels EM, Verstraeten N, Michiels J. 2019. General mechanisms leading to persister formation and awakening. *Trends Genet* 35:401–411.
2. Dewachter L, Fauvart M, Michiels J. 2019. Bacterial heterogeneity and antibiotic survival: Understanding and combatting persistence and heteroresistance. *Mol Cell* 76:255–267.
3. Fauvart M, de Groote VN, Michiels J. 2011. Role of persister cells in chronic infections: Clinical relevance and perspectives on anti-persister therapies. *J Med Microbiol* 60:699–709.
4. Fisher RA, Gollan B, Helaine S. 2017. Persistent bacterial infections and persister cells. *Nat Rev Microbiol* 15:453–464.
5. Lafleur MD, Qi Q, Lewis K. 2010. Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. *Antimicrob Agents Chemother* 54:39–44.
6. Schumacher MA, Balani P, Min J, Chinnam NB, Hansen S, Vulić M, Lewis K, Brennan RG.

2015. HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature 524:59–66.
7. Windels EM, Michiels JE, Fauvart M, Wenseleers T, Van den Bergh B, Michiels J. 2019. Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. ISME J 13:1239–1251.
8. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shores N, Balaban NQ. 2017. Antibiotic tolerance facilitates the evolution of resistance. Science 355:826–830.
9. Sebastian J, Swaminath S, Nair RR, Jakkala K, Pradhan A, Ajitkumar P. 2017. De novo emergence of genetically resistant mutants of Mycobacterium tuberculosis from the persistence phase cells formed against antituberculosis drugs in vitro. Antimicrob Agents Chemother 61:e01343-16.
10. Liu J, Gefen O, Ronin I, Bar-Meir M, Balaban NQ. 2020. Effect of tolerance on the evolution of antibiotic resistance under drug combinations. Science 367:200–204.
11. Shan Y, Gandt AB, Rowe SE, Deisinger JP, Conlon BP, Lewis K. 2017. ATP-dependent persister formation in *Escherichia coli*. mBio 8:e02267-16.
12. Manuse S, Shan Y, Canas-Duarte SJ, Bakshi S, Sun WS, Mori H, Paulsson J, Lewis K. 2021. Bacterial persisters are a stochastically formed subpopulation of low-energy cells. PLoS Biol 19:e3001194.
13. Kwan BW, Valenta JA, Benedik MJ, Wood TK. 2013. Arrested protein synthesis increases persister-like cell formation. Antimicrob Agents Chemother 57:1468–1473.
14. Cheverton AM, Gollan B, Przydacz M, Wong CT, Mylona A, Hare SA, Helaine S. 2016. A *Salmonella* toxin promotes persister formation through acetylation of tRNA. Mol Cell 63:86–96.
15. Amato SM, Orman MA, Brynildsen MP. 2013. Metabolic control of persister formation in *Escherichia coli*. Mol Cell 50:475–487.
16. Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, McKinney JD. 2013. Dynamic persistence of antibiotic-stressed *Mycobacteria*. Science 339:91–95.
17. Allison KR, Brynildsen MP, Collins JJ. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216–220.
18. Wilmaerts D, Dewachter L, De Loose P-J, Bollen C, Verstraeten N, Michiels J. 2019. HokB monomerization and membrane repolarization control persister awakening. Mol Cell 75:1031–1042.
19. Pu Y, Li Y, Jin X, Tian T, Ma Q, Zhao Z, Lin S, Chen Z, Li B, Yao G, Leake MC, Lo C-J, Bai F. 2019. ATP-dependent dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance. Mol Cell 73:143–156.
20. Lewis K. 2010. Persister cells. Annu Rev Microbiol 64:357–72.
21. Völzing KG, Brynildsen MP. 2015. Stationary-phase persisters to ofloxacin sustain DNA damage and require repair systems only during recovery. mBio 6:e00731–e00746.

- 783 22. Goormaghtigh F, Van Melderden L. 2019. Single-cell imaging and characterization of
784 *Escherichia coli* persister cells to ofloxacin in exponential cultures. Sci Adv 5:eaav9462.
- 785 23. Mok WWK, Brynildsen MP. 2018. Timing of DNA damage responses impacts persistence
786 to fluoroquinolones. Proc Natl Acad Sci U S A 115:e6301–e6309.
- 787 24. Murawski A, Brynildsen MP. 2021. Ploidy is an important determinant of
788 fluoroquinolone persister survival. Curr Biol 31:1–12.
- 789 25. White MA, Azeroglu B, Lopez-Vernaza MA, Hasan AMM, Leach DRF. 2018. RecBCD
790 coordinates repair of two ends at a DNA double-strand break, preventing aberrant
791 chromosome amplification. Nucleic Acids Res 46:6670–6682.
- 792 26. Truglio JJ, Croteau DL, van Houten B, Kisker C. 2006. Prokaryotic nucleotide excision
793 repair: The UvrABC system. Chem Rev 106:233–252.
- 794 27. Dhawale A, Bindal G, Rath D, Rath A. 2021. DNA repair pathways important for the
795 survival of *Escherichia coli* to hydrogen peroxide mediated killing. Gene 768:145297.
- 796 28. Grafstrom RH. 1986. The repair of pyrimidine dimers via a DNA-glycosylase mechanism.
797 Basic Life Sci 38:281–286.
- 798 29. Schallow BJ, Courcelle CT, Courcelle J. 2012. Mfd is required for rapid recovery of
799 transcription following UV-induced DNA damage but not oxidative DNA damage in
800 *Escherichia coli*. J Bacteriol 194:2637–2645.
- 801 30. Adebali O, Chiou YY, Hu J, Sancar A, Selby CP. 2017. Genome-wide transcription-coupled
802 repair in *Escherichia coli* is mediated by the Mfd translocase. Proc Natl Acad Sci U S A
803 114:E2116–E2125.
- 804 31. Brégeon D, Doddridge ZA, You HJ, Weiss B, Doetsch PW. 2003. Transcriptional
805 mutagenesis induced by uracil and 8-oxoguanine in *Escherichia coli*. Mol Cell 12:959–
806 970.
- 807 32. Mellon I, Hanawalt PC. 1989. Induction of the *Escherichia coli* lactose operon selectively
808 increases repair of its transcribed DNA strand. Nature 342:95–98.
- 809 33. Park JS, Marr MT, Roberts JW. 2002. *E. coli* transcription repair coupling factor (Mfd
810 protein) rescues arrested complexes by promoting forward translocation. Cell 109:757–
811 767.
- 812 34. Selby CP, Sancar A. 1993. Molecular mechanism of transcription-repair coupling. Science
813 260:53–58.
- 814 35. Ragheb MN, Thomason MK, Hsu C, Nugent P, Gage J, Samadpour AN, Kariisa A, Merrikh
815 CN, Miller SI, Sherman DR, Merrikh H. 2019. Inhibiting the evolution of antibiotic
816 resistance. Mol Cell 73:157–165.
- 817 36. Portman JR, Brouwer GM, Bollins J, Savery NJ, Strick TR. 2021. Cotranscriptional R-loop
818 formation by Mfd involves topological partitioning of DNA. Proc Natl Acad Sci
819 <https://doi.org/10.1073/pnas.2019630118>.
- 820 37. Epshtein V, Kamarthapu V, McGary K, Svetlov V, Ueberheide B, Proshkin S, Mironov A,
821 Nudler E. 2014. UvrD facilitates DNA repair by pulling RNA polymerase backwards.

822 Nature 505:372–377.

823 38. Kamarthapu V, Epshtein V, Benjamin B, Proshkin S, Mironov A, Cashel M, Nudler E. 2016.
824 ppGpp couples transcription to DNA repair in *E. coli*. Science 352:993–996.

825 39. Kang J, Blaser MJ. 2006. UvrD helicase suppresses recombination and DNA damage-
826 induced deletions. J Bacteriol 188:5450–5459.

827 40. Matson SW, Robertson AB. 2006. The UvrD helicase and its modulation by the mismatch
828 repair protein MutL. Nucleic Acids Res 34:4089–4097.

829 41. Michel B, Sinha AK, Leach DRF. 2018. Replication fork breakage and restart in *Escherichia*
830 *coli*. Microbiol Mol Biol Rev 82:e00013-18.

831 42. Bidnenko V, Lestini R, Michel B. 2006. The *Escherichia coli* UvrD helicase is essential for
832 Tus removal during recombination-dependent replication restart from Ter sites. Mol
833 Microbiol 62:382–396.

834 43. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen MP,
835 Bumann D, Camilli A, Collins JJ, Dehio C, Fortune S, Ghigo JM, Hardt WD, Harms A,
836 Heinemann M, Hung DT, Jenal U, Levin BR, Michiels J, Storz G, Tan MW, Tenson T, Van
837 Melder L, Zinkernagel A. 2019. Definitions and guidelines for research on antibiotic
838 persistence. Nat Rev Microbiol 17:441–448.

839 44. Theodore A, Lewis K, Vulić M. 2013. Tolerance of *Escherichia coli* to fluoroquinolone
840 antibiotics depends on specific components of the SOS response pathway. Genetics
841 195:1265–1276.

842 45. Norman TM, Lord ND, Paulsson J, Losick R. 2015. Stochastic switching of cell fate in
843 microbes. Annu Rev Microbiol 69:381–403.

844 46. Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG,
845 Alon U. 2006. A comprehensive library of fluorescent transcriptional reporters for
846 *Escherichia coli*. Nat Methods 3:623–628.

847 47. Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC. 2012. Oxidation of the guanine
848 nucleotide pool underlies cell death by bactericidal antibiotics. Science 336:315–319.

849 48. Hong Y, Zeng J, Wang X, Drlica K, Zhao X. 2019. Post-stress bacterial cell death mediated
850 by reactive oxygen species. Proc Natl Acad Sci U S A 116:10064–10071.

851 49. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. 2007. A common mechanism
852 of cellular death induced by bactericidal antibiotics. Cell 130:797–810.

853 50. Schmidt Grant S, Kaufmann BB, Chand NS, Haseley N, Hung DT. 2012. Eradication of
854 bacterial persisters with antibiotic-generated hydroxyl radicals. Proc Natl Acad Sci
855 109:12147–12152.

856 51. Dwyer DJ, Kohanski MA, Collins JJ. 2009. Role of reactive oxygen species in antibiotic
857 action and resistance. Curr Opin Microbiol 12:482–489.

858 52. Gruber CC, Walker GC. 2018. Incomplete base excision repair contributes to cell death
859 from antibiotics and other stresses. DNA Repair (Amst) 71:108–117.

- 860 53. Neeley WL, Essigmann JM. 2006. Mechanisms of formation, genotoxicity, and mutation
861 of guanine oxidation products. *Chem Res Toxicol* 19:491–505.
- 862 54. Dalhus B, Laerdahl JK, Backe PH, Bjørås M. 2009. DNA base repair - Recognition and
863 initiation of catalysis. *FEMS Microbiol Rev*.
- 864 55. Clauson CL, Saxowsky TT, Doetsch PW. 2010. Dynamic flexibility of DNA repair pathways
865 in growth arrested *Escherichia coli*. *DNA Repair (Amst)* 9:842–847.
- 866 56. Pani B, Nudler E. 2017. Mechanistic insights into transcription coupled DNA repair. *DNA*
867 *Repair (Amst)* 56:42–50.
- 868 57. Brandi G, Salvaggio L, Cattabeni F, Cantoni O, Mortelmans KE. 1991. Cytocidal and
869 filamentous response of *Escherichia coli* cells exposed to low concentrations of
870 hydrogen peroxide and hydroxyl radical scavengers. *Environ Mol Mutagen* 18:22–27.
- 871 58. Luria SE, Delbrück M. 1943. Mutations of bacteria from virus sensitivity to virus
872 resistance. *Genetics* 28:491–511.
- 873 59. William A. Rosche, Patricia L. Foster. 2000. Determining mutation rates in bacterial
874 populations. *Methods* 20:4–17.
- 875 60. Pope CF, O’Sullivan DM, McHugh TD, Gillespie SH. 2008. A practical guide to measuring
876 mutation rates in antibiotic resistance. *Antimicrob Agents Chemother* 52:1209–1214.
- 877 61. Ukkivi K, Kivisaar M. 2018. Involvement of transcription-coupled repair factor Mfd and
878 DNA helicase UvrD in mutational processes in *Pseudomonas putida*. *DNA Repair (Amst)*
879 <https://doi.org/10.1016/j.dnarep.2018.09.011>.
- 880 62. Menecier S, Coste G, Servant P, Bailone A, Sommer S. 2004. Mismatch repair ensures
881 fidelity of replication and recombination in the radioresistant organism *Deinococcus*
882 *radiodurans*. *Mol Genet Genomics* 272:460–469.
- 883 63. Barrett TC, Mok WWK, Murawski AM, Brynildsen MP. 2019. Enhanced antibiotic
884 resistance development from fluoroquinolone persists after a single exposure to
885 antibiotic. *Nat Commun* 10.
- 886 64. Hiom K. 2009. DNA Repair: Common approaches to fixing double-strand breaks. *Curr*
887 *Biol* 19:532–525.
- 888 65. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. 2013. Killing by bactericidal antibiotics
889 does not depend on reactive oxygen species. *Science* 339:1213–1216.
- 890 66. Giroux X, Su WL, Bredeche MF, Matic I. 2017. Maladaptive DNA repair is the ultimate
891 contributor to the death of trimethoprim-treated cells under aerobic and anaerobic
892 conditions. *Proc Natl Acad Sci U S A* <https://doi.org/10.1073/pnas.1706236114>.
- 893 67. Veening J-W, Smits WK, Kuipers OP. 2008. Bistability, Epigenetics, and Bet-Hedging in
894 Bacteria. *Annu Rev Microbiol* 62:193–210.
- 895 68. Pribis JP, García-Villada L, Zhai Y, Lewin-Epstein O, Wang AZ, Liu J, Xia J, Mei Q, Fitzgerald
896 DM, Bos J, Austin RH, Herman C, Bates D, Hadany L, Hastings PJ, Rosenberg SM. 2019.
897 Gamblers: An antibiotic-induced evolvable cell subpopulation differentiated by reactive-
898 oxygen-induced general stress response. *Mol Cell* 74:785–800.

899 69. Ryder VJ, Chopra I, Neill O. 2012. Increased mutability of *Staphylococci* in biofilms as a
900 consequence of oxidative stress. PLoS One 7:e47695.

901 70. Han J, Sahin O, Barton YW, Zhang Q. 2008. Key role of Mfd in the development of
902 fluoroquinolone resistance in *Campylobacter jejuni*. PLoS Pathog 4:e1000083.

903 71. Gómez-Marroquín M, Martin HA, Pepper A, Girard ME, Kidman AA, Vallin C, Yasbin RE,
904 Pedraza-Reyes M, Robleto EA. 2016. Stationary-phase mutagenesis in stressed bacillus
905 subtilis cells operates by Mfd-dependent mutagenic pathways. Genes (Basel)
906 <https://doi.org/10.3390/genes7070033>.

907 72. Wimberly H, Shee C, Thornton PC, Sivaramakrishnan P, Rosenberg SM, Hastings PJ.
908 2013. R-loops and nicks initiate DNA breakage and genome instability in non-growing
909 *Escherichia coli*. Nat Commun 4:2115.

910 73. Torkelson J, Harris RS, Lombardo MJ, Nagendran J, Thulin C, Rosenberg SM. 1997.
911 Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies
912 recombination-dependent adaptive mutation. EMBO J 16:3303–3311.

913 74. Vincent MS, Uphoff S. 2020. Bacterial phenotypic heterogeneity in DNA repair and
914 mutagenesis. Biochem Soc Trans.

915 75. Pomerantz RT, O'Donnell M. 2010. What happens when replication and transcription
916 complexes collide? Cell Cycle 9:2537–2543.

917 76. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M,
918 Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene
919 knockout mutants: the Keio collection. Mol Syst Biol 2.

920 77. Vicente NB, Diaz Zamboni JE, Adur JF, Paravani E V., Casco VH. 2007. Photobleaching
921 correction in fluorescence microscopy images. J Phys Conf Ser 90:012068.

922 78. Peccia J, Hernandez M. 2002. Rapid immunoassays for detection of UV-induced
923 cyclobutane pyrimidine dimers in whole bacterial cells. Appl Environ Microbiol 68:2542–
924 2549.

925