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Effects of cephalixin treatment on DNA replication dynamics in *Escherichia coli*

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ABSTRACT

Background

In bacteria, DNA replication and chromosome segregation are tightly coordinated with cell division. The replication run-out method, which combines inhibition of transcription initiation by rifampicin with inhibition of cell division by cephalixin, is widely used to analyse replication dynamics by allowing ongoing replication forks to complete in the absence of new initiations. While this approach is generally robust, the physiological consequences of the individual components of the method, particularly cephalixin, are less well characterised.

Results

Here, we investigated the effects of cephalexin treatment on DNA replication in *Escherichia coli*. Cephalexin treatment resulted in rapid filamentation and, when applied alone, led to extensive cell lysis. Concomitantly, a subpopulation of cells displayed an increase in the number of replisome-associated DnaN foci, which was accompanied by amplification of both origin- and terminus-proximal chromosomal loci. Genetic analysis demonstrated that this response was largely dependent on the replication initiator DnaA, whereas mutants impaired in replication restart pathways did not show a corresponding reduction, arguing against a major contribution from pathways such as recombination-dependent replication. Importantly, co-treatment with rifampicin largely suppressed the increase in replication activity observed following cephalexin treatment and strongly reduced cell lysis, revealing a physiological antagonism between the two drugs under these conditions.

Conclusions

Our findings show that cephalexin treatment alone is not physiologically neutral with respect to DNA replication and can result in DnaA-dependent replication activity in a subset of cells. While the classical rifampicin-cephalexin replication run-out approach largely mitigates these effects, residual heterogeneity remains and may be exacerbated in sensitised genetic backgrounds. These results highlight the importance of considering antibiotic-induced physiological responses, including antagonistic drug interactions, when interpreting replication phenotypes in both single- and combination-treatment experiments.

BACKGROUND

Cephalexin is an antibiotic that belongs to the β -lactam family. As a first-generation cephalosporin, it shows strong activity against Gram-positive bacteria like *Staphylococcus* and *Streptococcus* species, as well as some Gram-negative bacteria, including *E. coli* [1]. It has been used clinically for decades to treat bacterial infections [2]. Especially infections of the skin, such

as impetigo, respiratory tract and urinary tract often respond well to treatment with cephalexin [1, 3].

The antimicrobial activity of cephalexin is achieved by the inhibition of bacterial cell wall synthesis, both in Gram-positive and Gram-negative bacteria [1]. It specifically targets the final step of peptidoglycan synthesis, a main component of bacterial cell wall structure [4]. Cephalexin acylates the active-site serine of penicillin binding proteins (PBPs) such as FtsI. Specifically, it disrupts cell wall formation by interacting with PBPs, particularly PBP3 [5]. These PBPs are crucial enzymes responsible for cross-linking peptidoglycan polymers in the bacterial cell wall [6]. Cephalexin prevents this cross-linking process, leading to cell wall instability and, under many conditions, cell death [5]. However, while PBP2 are the main enzymes used for elongation of bacterial cells, PBP3, which is also known as FtsI, acts within the divisome. For this reason cephalexin triggers a strong inhibition of septum formation, thereby preferentially targeting the late-stage cell division processes [7]. Although cephalexin is frequently described simply as a cell division or septum formation inhibitor, it is fundamentally a β -lactam antibiotic targeting PBP3-mediated peptidoglycan synthesis. The inhibition of septum formation is therefore a downstream consequence of its primary mode of action. In addition to its clinical use, cephalexin also has been used for many years for molecular cell biology purposes, specifically in so-called replication run-out experiments due to its ability to inhibit the formation of a septum.

Many bacteria harbour a single circular chromosome. DNA replication is initiated at *oriC* and proceeds bidirectionally until the two replication forks meet and fuse within a specialised termination region opposite *oriC* [8]. During rapid growth, bacteria such as *E. coli* cells can have overlapping rounds of DNA replication, which means that new rounds of replication are initiated before the previous rounds have completed, resulting in multiple replication forks operating simultaneously on a single chromosome [9].

Multi-fork replication allows bacteria to achieve generation times that are significantly shorter than the time required to complete a single round of chromosome replication. In LB broth,

E. coli MG1655 cells will double approximately every 20 min [10, 11], while genome duplication takes approximately 40–50 min [12]. The number of ongoing rounds of replication in a cell reflects its growth rate and nutrient conditions, making it an important parameter for understanding bacterial cell cycle dynamics. Therefore, the determination of how many ongoing rounds of replication exist in growing cells has been a fundamental question in bacterial physiology, and the replication run-out procedures were established in the 1980s to address this question.

Replication run-out procedures are based on the fact that firing of *oriC* can be inhibited without affecting ongoing rounds of DNA replication. This can be achieved with temperature-sensitive versions of the main initiator protein DnaA. Because a threshold concentration of DnaA is required to initiate synthesis at *oriC* [13], some molecules that block either transcription or translation will efficiently block *oriC* activity. In 1986 the Skarstad lab demonstrated that one such molecule is the antibiotic rifampicin [14]. Rifampicin acts by inhibiting RNA polymerase [15, 16], thereby blocking transcription. As this prevents the generation of the additional DnaA molecules necessary to initiate new rounds of DNA replication, firing of *oriC* is inhibited. It does not, however, interfere with already ongoing rounds of DNA replication, which continue the duplication process [14, 17]. This selective inhibition ensures that firing of *oriC* stops while all active replication forks at the time of drug addition will finish replicating [18].

The inhibition of septum formation by cephalixin was then used to further improve this methodology. By combining both rifampicin to block new rounds of synthesis while letting all ongoing rounds complete synthesis and cephalixin to inhibit cell division, Løbner-Olesen and colleagues generated the “replication run-out” method [19]. When *E. coli* cells are treated with both rifampicin and cephalixin for 3–4 h, they reach the “run-out” state in which filamentous cells contain fully replicated chromosomes in numbers that directly correspond to the number of origins of replication (*oriC*) present at the time the drugs were added [14, 18, 19]. Flow cytometry is then used to measure the DNA content of these cells, providing a DNA histogram with distinct

peaks representing the different numbers of chromosomes [20]. While the method results in visibly segregated chromosomes in wild type cells [21], it also can be used in situations where segregation cannot be completed. The use of DNA histograms does not only allow the estimation of chromosome equivalents, but can also be used to demonstrate that chromosome duplication remains uncompleted in specific mutants [22, 23].

The rifampicin-cephalexin run-out technique is a valuable method that has been widely used for determining the number of replication origins and ongoing replication forks in specific *E. coli* mutants [24–27], but also has been used in other bacteria, such as *Vibrio cholerae* [28], *Caulobacter crescentus* [29] and others, providing important fundamental insights into bacterial cell cycle dynamics and replication control. As useful as the method has proven, it remains important to consider the potential pleiotropic effects of drugs such as rifampicin and cephalexin when interpreting experimental outcomes: sometimes the original functions of these compounds are lost in the context of a specific study, which should be considered particularly in sensitised mutant backgrounds. Although the replication run-out technique has been widely applied, cephalexin retains its broader physiological effects as a β -lactam antibiotic, including induction of filamentation and bulge-mediated lysis [30–32], features that are not always explicitly discussed in the context of replication studies.

As part of the review process for a recent manuscript investigating DNA replication termination in *E. coli* MG1655 [33], a control experiment was suggested in which cephalexin would be used to inhibit cell division while monitoring DNA replication patterns. When we performed this experiment, we observed unexpected changes in replication-associated markers and pronounced alterations in cell morphology. These findings prompted a more systematic analysis of the physiological consequences of cephalexin treatment, both alone and in combination with rifampicin. The results of these investigations are presented in this study.

MATERIALS AND METHODS

Strains, media and general methods

Escherichia coli K-12 strain MG1655 used in this study was obtained from existing laboratory stocks derived from standard laboratory collections. All strain derivatives used in this study are listed in Table 1. Strains were constructed via *P1vir* transductions [34] or by single-step gene disruptions [35]. Rich broth (LB) and agar was used in Miller composition and contained 1% tryptone (Bacto™, BD Biosciences), 0.5% yeast extract (Bacto™, BD Biosciences), and 1% NaCl (Sigma Aldrich) [36]. The pH was adjusted to 7.4. For plates agar was added to a final concentration of 1%. M9 minimal medium was purchased as a 5 × concentrated stock (Sigma-Aldrich), which contained 15 g/L KH₂PO₄, 64 g/L Na₂HPO₄, 2.5 g/l NaCl and 5.0 g/L NH₄Cl. Before use, MgSO₄, CaCl₂ and glucose were added from sterile-filtered stock solutions to final concentrations of 2 mM, 0.1 mM and 0.2%, respectively, according to the manufacturer's recommendation.

Single-image microscopy of *E. coli* cells with fluorescently tagged proteins

For imaging of cells with fluorescently labelled proteins, fresh overnight cultures of the *E. coli* strains of interest were diluted 100-fold in LB broth (Miller composition) and incubated with vigorous aeration at 37 °C until OD₆₀₀ reached 0.2. For the Cas1-LFP-Cas2 expression system [37], ampicillin was added to the culture at a final concentration of 50 µg/ml to maintain plasmid selection. For this system, upon reaching an OD₆₀₀ of 0.2 L-arabinose (Sigma-Aldrich) was added to a final concentration of 0.1% to induce expression of Cas1-LFP-Cas2 from the *paraBAD* promoter [37]. Cells were then incubated for a further 60 min. For samples treated with either cephalixin or saccharin, the drug was added to the final concentration indicated in the figures when the OD₆₀₀ reached 0.2, at the same time as the arabinose was added. After the 60 min incubation, 1 µl aliquots of treated and mock-treated samples were directly pipetted onto agarose pads and air-dried. For generation of pads, a 65 µl (15 × 16 mm) GeneFrame (Thermo Scientific)

was added to a conventional microscopy slide. 1% SeaKem LE agarose (Lonza) was added to 1 × M9 minimal medium (diluted from a 5 × stock, Sigma-Aldrich) and heated until the agarose was completely dissolved. 95 µl of the solution was added into the GeneFrame chamber and the chamber sealed immediately with a conventional microscopy slide. Once set, the top slide was removed and the agarose pad air-dried for a maximum of 5 min at 42 °C and used immediately. Once the sample was added and air-dried, the GeneFrame chamber was sealed by adding a 22 × 22 mm cover slip. Visualisation was done using a Ti-U inverted microscope (Nikon) with a CFI Plan Fluor DLL 100 × objective (Nikon) and an ORCA Flash 4.0 LT plus camera (Hamamatsu). Phase contrast images were taken using a pE-100 single LED wavelength source (CoolLED). For fluorescence, the pE-4000 illumination system (CoolLED) was used. The relevant filters for visualisation of Hoechst 33342, eCFP, eYFP/YPet and mCherry were Zeiss filter sets 47 (eCFP) and 46 (eYFP/YPet), DAPI-50LP-A (Hoechst 33342) and Nikon TXRED-A-Basic (mCherry). Images were captured using the NIS Elements-BR software V4.51 (Nikon). Standard exposure settings were 1 second at 75% intensity for Cas1-linker-eYFP-Cas2, 2 seconds at 75% intensity for YPet-DnaN and eCFP-TetR, and 3 seconds at 75% intensity for mCherry-LacI. Postprocessing, such as cropping and rotating, was performed in Adobe Photoshop CC (V26.8.1). All image processing was performed using standard procedures. Brightness and contrast adjustments were applied uniformly across entire images. No image manipulations that could alter the interpretation of the data were performed. For all single image microscopy experiments a minimum of two biological replicates were performed, with a minimum of 3 individual frames being recorded per condition and experiment. For foci numbers, foci in cells were counted manually from two separate experiments, using a minimum of three individual frames per experiment. Counting was performed blind with respect to strain and treatment. The total number of cells counted is stated in the relevant figures. All Violin Plots were generated using SigmaPlot (V16, Grafiti LLC). Statistical comparisons of all foci counts were performed using non-parametric Mann-Whitney U tests implemented in SigmaPlot; *p*-values are indicated in the figures. In some

experiments, the number of cells analysed was lower because antibiotic treatment caused extensive filamentation and cell lysis, which reduced the number of analysable cells per microscopic field. All clearly identifiable cells in the recorded images were manually counted to maximise sample size. In experiments where cell numbers were lower, differences between conditions were large and reproducible across independent experiments, allowing reliable statistical analysis despite reduced sample size.

Time-lapse microscopy

Fresh overnight cultures of the *E. coli* strains of interest were diluted 100-fold in fresh LB broth (Miller composition) and incubated with vigorous aeration at 37 °C until reaching an OD₆₀₀ of 0.2. 1 µl of the sample was pipetted onto an agarose pad and air-dried. For generation of pads 65 µl (15 × 16mm) GeneFrames (Thermo Scientific™) were used. 1% of SeaKem LE agarose (Lonza) was added to LB broth (Miller composition) and heated until the agarose was completely dissolved. If required, cephalixin was added to the molten agarose solution at the required concentration. 95 µl of the solution was added into a GeneFrame and the chamber sealed immediately with a conventional microscopy slide. Once the agarose had set, the top slide was removed and the pad air-dried for 12 min at room temperature and used immediately. Once the sample was added and air-dried, the GeneFrame chamber was sealed by adding a 22 × 22 mm cover slip. Cells were visualised using the Ti-U system described above. The temperature was maintained at 37 °C using an environmental chamber (Digital Pixel). Time-lapse stacks were captured using the NIS Elements-BR software V4.51 (Nikon). Postprocessing of images, such as cropping and rotating, was performed in Adobe Photoshop CC (V26.8.1).

Single-image microscopy using temperature-sensitive alleles

For visualisation of fluorescently tagged DnaN protein in the *dnaA46* background, cells were grown at the permissive temperature of 30 °C to an OD₆₀₀ of 0.2. 1 ml aliquots were then transferred into pre-warmed glass tubes and incubated either at 30 °C or the restrictive

temperature of 42 °C. Cells were incubated for 30 more minutes and then either mock-treated or the relevant drug added to the concentration indicated in the figures. Visualisation was otherwise done as described above.

Cell length analysis

For the analysis of cell lengths in brightfield images, free plugin MicrobeJ [38] was used in Fiji/ImageJ [39]. MicrobeJ automatic detection was performed for cell segmentation and cell length analysis. Following the automatic detection, some manual adjustments were used to verify cell outline detection and, if necessary, adjustments were made to restrict the analysis to individual cells. Cell length data were then copied to SigmaPlot (V16, Grafiti LLC). Data representation was achieved by using the Violin plot function on the raw cell length data within SigmaPlot. Statistical comparisons of all cell length data were performed using non-parametric Mann–Whitney U tests implemented in SigmaPlot (V16, Grafiti LLC); *p*-values are indicated in the figures. In some experiments, the number of cells analysed was lower because antibiotic treatment caused extensive filamentation and cell lysis, which reduced the number of cells per microscopic field that could be analysed. In these cases, all clearly identifiable cells in the recorded images were included to maximise sample size. Despite the lower cell numbers in some conditions, differences between treatments were large and reproducible across independent experiments, allowing reliable statistical comparisons.

RESULTS

Effects of cephalexin treatment on cell morphology in *E. coli*

To characterise the physiological consequences of cephalexin treatment under our experimental conditions, exponentially growing *E. coli* MG1655 cells were exposed to cephalexin and monitored by time-lapse microscopy. *E. coli* MG1655 cells were grown in LB broth (Miller composition) to an A_{600} of 0.2. The cells were then transferred to a microscopy slide with a LB agarose pad (see Materials and Methods) containing 40 µg/ml cephalexin. The temperature was maintained using

an environmental chamber and microscopy images taken in regular intervals. Cell division rapidly ceased and cells started to filament (Fig. 1A), an effect reported before [32, 40]. After 60 min of incubation the average cell size of the mock-treated sample was $\sim 3\ \mu\text{m}$ while the average filament length observed after treatment for the same period with either 10 or 40 $\mu\text{g/ml}$ cephalixin was ~ 15 and $\sim 18\ \mu\text{m}$, respectively (Fig. 1B), showing that within a period that allows a maximum of 3 doublings, the filaments grew to between 5 and $6 \times$ their length, with multiple filaments reaching 40 μm and more (Fig. 1B). The filaments showed multiple Z rings (Fig. 1C), which was shown previously [21]. Consistent with PBP3 inhibition acting downstream of FtsZ [41], multiple Z-rings remained detectable during cephalixin treatment.

We observed the first membrane bulges at the mid-cell position after approximately 30 min, with individual cells lysing only a few minutes later, leaving behind a “ghost” (Fig. 1A; white arrow). Although lysis events could be detected at these early time points, their frequency increased markedly after ~ 50 min of treatment, consistent with previous reports at higher cephalixin concentrations [32]. By 70–80 min a substantial fraction of cells had lysed under our experimental conditions.

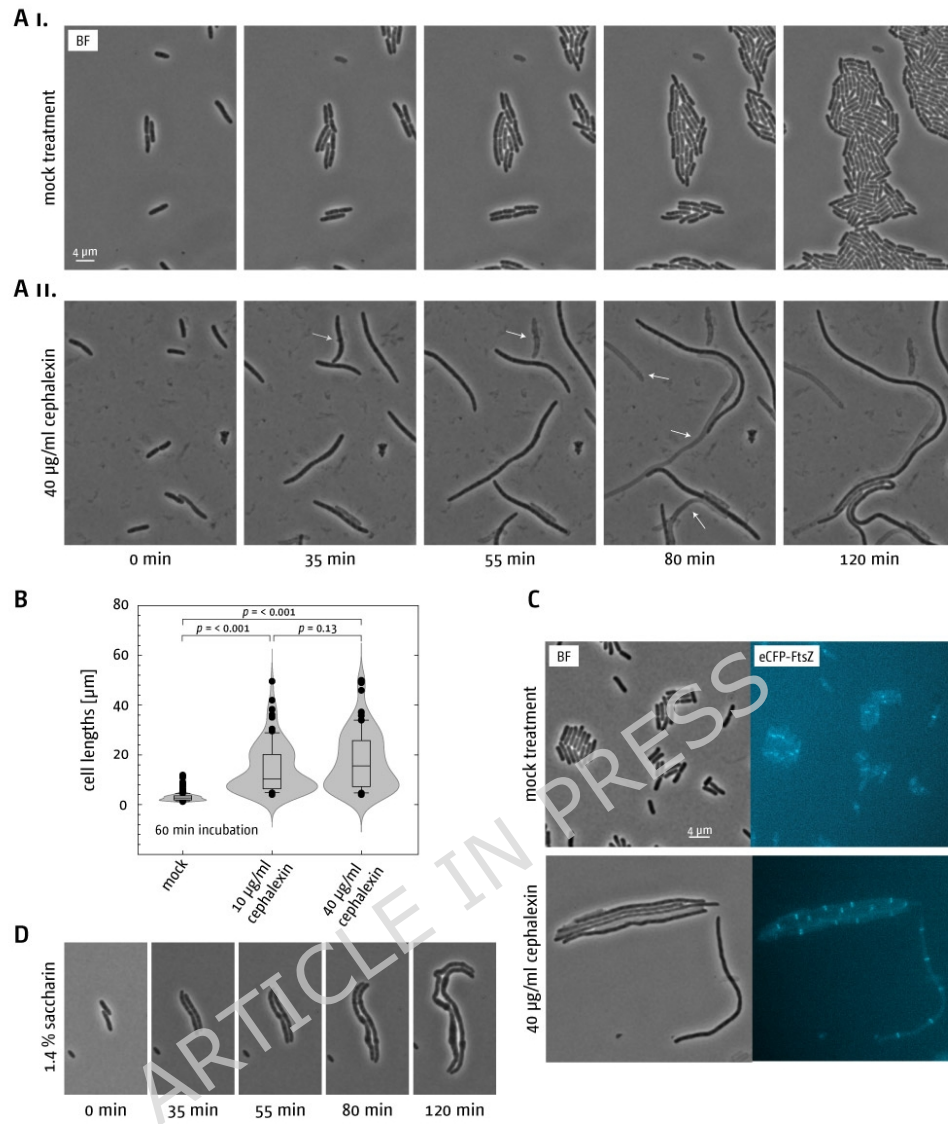


Figure 1. Impact of cephalaxin on growth and morphology of *E. coli*.

(A) Phase-contrast time-lapse images of MG1655 cells grown in LB (Miller) to $A_{600} = 0.2$ and transferred to LB agarose pads with or without 40 $\mu\text{g/ml}$ cephalaxin. Cells were imaged in an environmental chamber at 37 °C

for 120 min. Formation of a membrane bulge (grey arrow) followed by lysis and formation of a “ghost” (white arrows) is visible in cephalaxin-treated cells.

(B) Cell length analysis of cells mock-treated or treated with cephalaxin (10 or 40 $\mu\text{g/ml}$) for 60 min before imaging. Numbers of cells analysed: 682 (mock), 102 (10 $\mu\text{g/ml}$), 92 (40 $\mu\text{g/ml}$).

(C) Phase-contrast and fluorescence images of cells treated with 40 $\mu\text{g/ml}$ cephalaxin for 60 min. Septum formation was visualised using FtsZ-eCFP (strain RCe849; see Materials and Methods).

(D) Phase-contrast time-lapse images as in (A), but with 1.4% saccharin included in the agarose pad. Representative images from at least two biological replicates are shown.

Recently we analysed the effects of the artificial sweetener saccharin on bacterial cells [42]. We found that treatment of *E. coli* cells with saccharin concentrations between 1.4 and 2% caused morphological changes similar to the changes observed following treatment with cephalixin. Upon addition of saccharin cell division stops immediately and cells start to filament. However, filamentation is nowhere near as extensive as observed following cephalixin treatment. Saccharin-treated cells develop a clearly visible central bulge, and growing membrane bulges appear before cells start to lyse approximately 50–60 min into the experiment (Fig. 1D) [42]. Importantly, we found that in cells treated with saccharin, DNA synthesis continues, resulting in the filaments being filled with sites of ongoing synthesis, as visualised by using cells in which the β -sliding clamp, encoded by the *dnaN* gene, is tagged with a fluorescent protein (YPet-DnaN) [42]. Analysis of foci numbers revealed that most foci simply arise because of ongoing synthesis that is initiated at the origin. However, our analysis also showed that in a fraction of cells additional synthesis is triggered, either by the cellular stress response, some damage-induced DNA synthesis or both [42]. Given the comparable morphological responses, we wanted to investigate whether similar effects on DNA replication can be observed in cells treated with cephalixin.

Visualisation of DnaN foci in cephalixin-treated cells

To investigate the effect of cephalixin treatment on DNA replication, we grew cells to an A_{600} of 0.2 and then added cephalixin to a final concentration of 40 $\mu\text{g}/\text{ml}$ or mock-treated the culture and incubated for 60 min in the shaking water bath before visualising samples. In cells with overlapping rounds of synthesis, 6 replisomes traverse the chromosome. In the mock-treated culture the majority of cells show 3 (35% of cells) foci, with some showing 4 (24%), in line with overlapping rounds of DNA synthesis. 5 and 6 foci were only observed in a smaller number (12.5%) of cells (Fig. 2A & 2B). This is due to two reasons. Firstly, cultures incubated for 60 min after reaching an A_{600} of 0.2 are progressing into early stationary phase, which results in mildly

slower growth rates and fewer replisomes. In addition, the two replisomes are confined to a region around the mid-cell position that is close to the resolution limit of conventional wide-field fluorescence microscopy [43], which means that two replisomes can either form a single focus or two foci, depending on how far apart they are at the time of imaging [44]. Thus, even in cells with 6 replisomes we would only observe 3, 4 or 5 foci in many cells, depending on how many of the replisomes happen to be spatially separated.

Following treatment with 40 $\mu\text{g/ml}$ cephalixin, we observed filaments with a large number of foci. Quantification of foci numbers revealed a wide spread, with individual cells showing up to 70 foci. In $\sim 50\%$ of cells we counted up to 30 foci, in line with cells having undergone 2–3 chromosome duplication events, and some individual replisomes being resolved as individual foci. However, the remaining 50% of cells showed higher foci numbers, with some stretching all the way to over 70 foci (Fig. 2A & 2B).

Because of the complication of focus resolution as well as multiple rounds of divisions we also recorded foci numbers at 20 min intervals. Rather than incubating for 60 min, we visualised the first sample immediately. The higher number of foci (cf “mock” in Fig. 2B and “0 min” in Fig. 2C) fits well with a rapidly growing culture at an A_{600} of 0.2, while after incubation for 60 min growth starts to slow down as cells progress into early stationary phase. We showed before that for our experimental conditions at an A_{600} of 0.2 the doubling time in rich medium is ~ 20 min [11, 45]. At time 0 the median number of foci was 6 (Fig. 2C). 20 min later we observed a median number of foci of 13, more than double than what was observed at the 0 min time point. 20 min later, at 40 min, the median number of foci was 23, and after 60 min the number and distribution were the same as observed in our previous single time point experiments (Figs 2B & 2C). Thus, especially for 20 and 40 min we roughly see a doubling of the median number of foci, with a significant skew towards higher foci numbers (up to 50 foci after 20 min, 64 after 40 min and 72 after 60 min). These foci numbers are in line with a doubling of foci numbers every 20 min in most cells, but with a fraction of cells showing significantly larger foci numbers. This suggests

heterogeneous behaviour in the population, rather than simple uniform doubling. Some cells are accumulating foci faster than expected, and the population is becoming increasingly heterogeneous over time. These results become even more significant if the progression into early stationary phase is taken into consideration, as this slows metabolic activity of cells down.

These results differ significantly from our observation made in cells treated with saccharin [42]. Following saccharin treatment, cessation of cell division is observed [42], similarly to treatment with cephalixin. Thus, if the number of foci per filament is just determined by the number of chromosome duplication events, then we should expect to see similar foci numbers. However, this was not observed. Following treatment with 1.4% saccharin we observed that 97% of cells had up to 24 foci (Fig. 2B), highlighting that foci numbers are significantly lower than observed following cephalixin treatment, despite cell division being blocked.

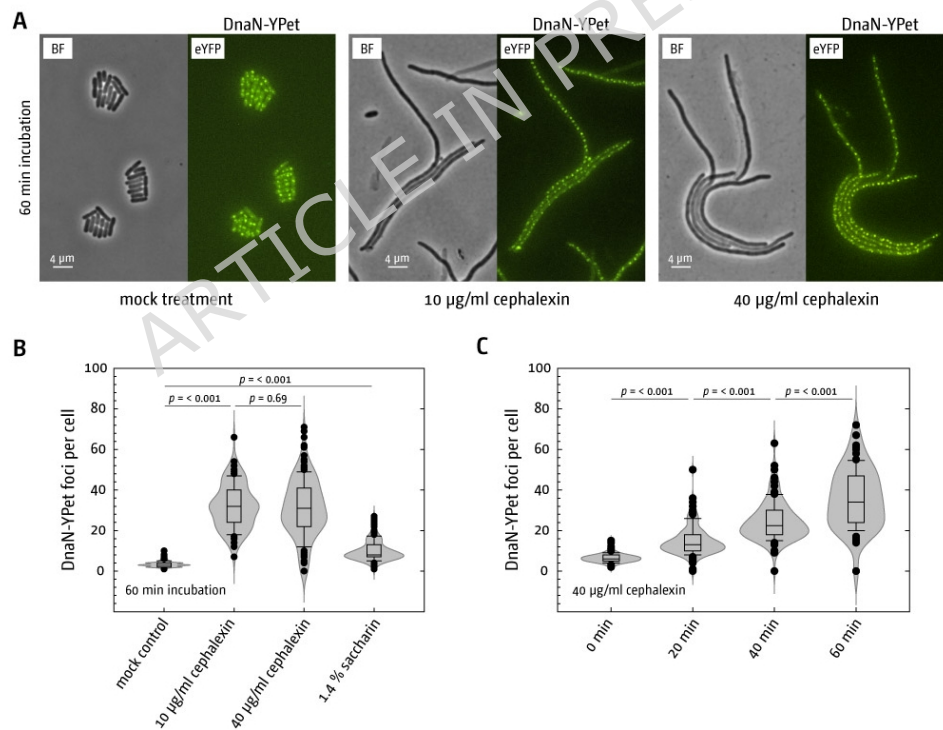


Figure 2. Replication dynamics in living cells following cephalixin treatment.

(A) Active replication complexes in MG1655 cells expressing YPet-DnaN (strain AS1062). Cells were grown in LB (Miller) to $A_{600} = 0.2$ and mock-treated or treated with cephalixin for 60 min at the concentrations indicated.

(B) Quantification of replisome foci per cell after treatment with cephalixin or saccharin. Foci were counted from two independent experiments using ≥ 3 random frames per replicate, and data were pooled. Numbers of cells analysed: 521 (mock), 223 and 212 (cephalexin), and 177 (saccharin). Saccharin data were re-plotted from de Dios *et al.* [42] for comparison.

(C) Time-dependent increase in replisome foci after cephalixin treatment. Cells were treated as in (A) and imaged at the time points indicated. Foci were quantified from two independent experiments as in (B). Numbers of cells analysed: 178 (0 min), 195 (20 min), 112 (40 min), and 97 (60 min).

For saccharin we also reported a concentration dependency: at higher concentrations of 2% saccharin, foci numbers are significantly increased [42]. This would not be expected if the only effect is the inhibition of cell division, as this effect is observed at the lower concentration of 1.4% saccharin. To look at any concentration dependency, and to also cover the lower cephalixin concentrations often used in experimental set-ups, we counted foci numbers in cells incubated for 60 min with 10 $\mu\text{g}/\text{ml}$ cephalixin, a concentration that is frequently used in replication run-out experiments [46–48]. In contrast to saccharin treatment [42], we found that foci numbers were remarkably similar following incubation with either 10 or 40 $\mu\text{g}/\text{ml}$ cephalixin for 60 min (Fig. 2A & 2B), with the median number of foci being identical for both concentrations (Fig. 2B).

Based on our data we concluded that the majority of foci following saccharin treatment can be explained simply by the absence of cell division, but the skew towards high foci numbers suggests that saccharin treatment also must trigger additional replication activity [42]. The higher foci numbers in cephalixin-treated cells suggest that even higher levels of replication activity must be triggered, at least in some cells. However, while the concentration-dependency of saccharin is in line with some damage-induced synthesis, such as break-induced replication, the lack of concentration-dependency for cephalixin, at least at the concentrations tested, points towards other explanations, such as over-initiation at the origin, or possibly the influence of a triggered stress response such as the SOS response. Some antibiotics trigger the SOS stress response very strongly, such as ciprofloxacin, a fluoroquinolone that targets DNA gyrase [49]. The SOS response is not as strongly induced in cephalixin-treated cells, but it was shown that levels are still elevated [40].

Chromosome replication dynamics in cephalixin-treated cells

One difficulty of the replication analysis is that replisomes can form either two separate foci or a single unresolved focus, depending on their distance. Given the extensive filamentation of the cells the large number of foci could, at least in part, be caused simply by more space allowing replisomes being further apart, allowing them to be resolved in our microscope set up. To avoid this problem, we used two different additional experimental systems to address whether more synthesis is triggered in cephalixin-treated cells, or whether the amounts are in line with cells simply not dividing.

Firstly, we visualised chromosome replication dynamics by using an *E. coli* strain in which the origin and terminus regions of the chromosome can be tracked by fluorescent repressor-operator systems (FROS) (Fig. 3A). Thus, the use of this strain allows the direct visualisation of the number of chromosomal origin and terminus regions, a scenario where the spatial resolution of foci does not apply. Secondly, we visualised Cas1-Cas2 complexes in cells treated with cephalixin. We recently generated a functional Cas1-Cas2 complex where Cas1 contains a linker, followed by an enhanced yellow fluorescent protein (eYFP). We call this Cas1-linker-eYFP-Cas2 complex Cas1-Cas2 in the rest of the text, for simplicity. We demonstrated recently that, if over-expressed in *E. coli* cells, Cas1-Cas2 localises into distinct foci in cells where DNA is being actively synthesised, allowing its use as a biomarker of active DNA synthesis [37]. Cas1-Cas2 foci numbers are lower than the number of replisome foci, suggesting that Cas1-Cas2 is not binding directly at forks, but instead to a substrate generated in cells that actively replicate their chromosome [37]. This system is extremely simple to use, as the fluorescent signal is strong and it can be added simply via an expression plasmid, allowing the rapid generation of a variety of different strains. In addition, we visualised chromosome content in cells using the DNA dye Hoechst 33342 (called “DNA” in the related figures for simplicity).

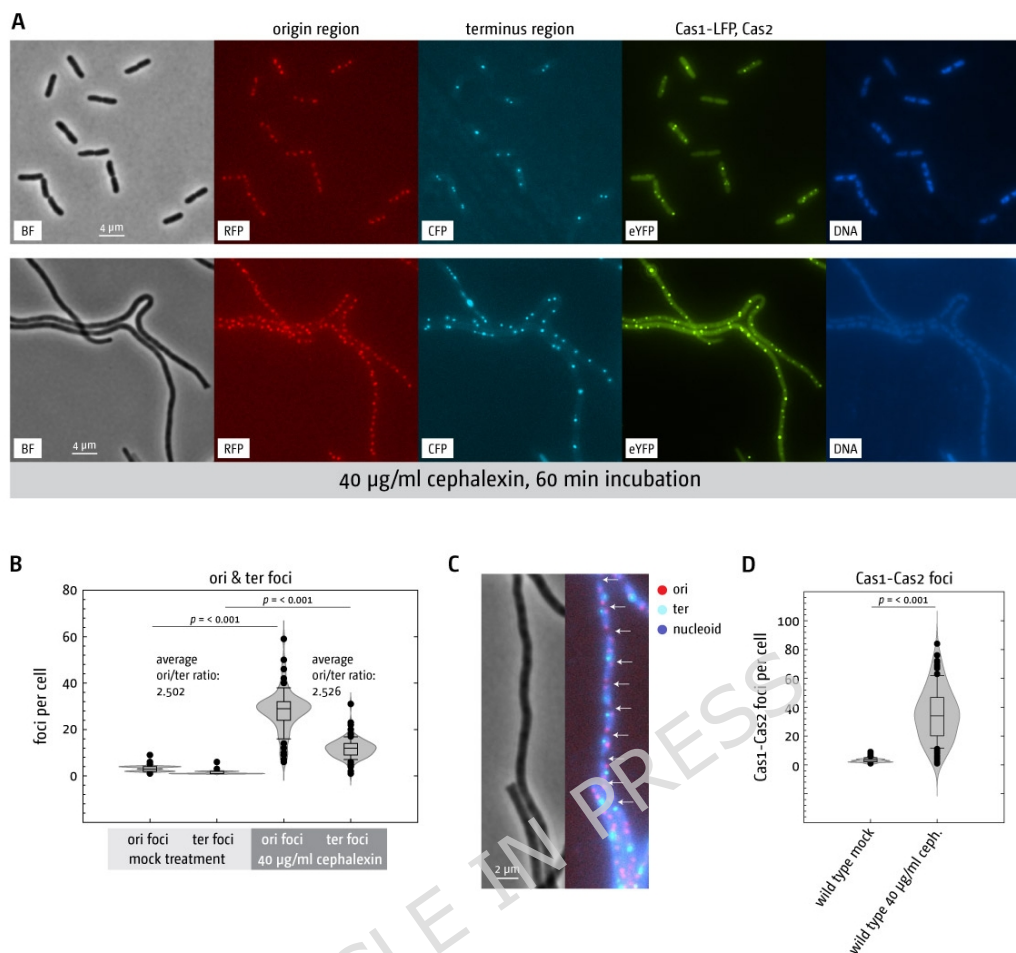


Figure 3. Chromosome dynamics in living cells following cephalixin treatment.

(A) Visualisation of chromosomal origin (red) and terminus (cyan) regions using fluorescent repressor–operator arrays (FROS), Cas1-Cas2 complexes (green), and DNA (Hoechst 33342; labelled as “DNA”, blue) in strain JD1841. Cells were grown in LB (Miller) to $A_{600} = 0.2$ and mock-treated or treated with 40 µg/ml cephalixin for 60 min. Untreated cells typically display 2–4 ori foci and 1–2 ter foci, consistent with overlapping rounds of replication.

(B) Quantification of origin and terminus foci per cell after cephalixin treatment. Foci were counted from two independent experiments using ≥ 3 random frames per replicate, and data were pooled. Numbers of cells analysed: 428 (mock) and 81 (cephalexin).

(C) Spatial distribution of ori and ter foci in filamentous cells treated with 40 µg/ml cephalixin for 60 min. Experiment as in (A); only ori, ter, and DNA channels are shown. Gaps between segregated nucleoid equivalents are indicated by white arrows.

(D) Quantification of Cas1–Cas2 foci after cephalixin treatment. Counting was performed as in (B). Numbers of cells analysed: 611 (mock wild type) and 115 (cephalexin-treated wild type).

For FROS, in the mock-treated control cells are able to start a maximum of three rounds of chromosomal replication within the 60 min period. With cell division not inhibited we can observe the expected 2–4 origin foci, with the terminus foci lagging one step behind in the duplication progression (1–2 foci). After treatment with cephalixin, we observed a significant amplification of both origin and terminus foci (Fig. 3A,3B), in line with the dramatic increase in the number of DnaN foci (Figs 2B). The median number of origin foci is increased from 3 to ~28, which would require just over 3 doublings. However, as for DnaN foci, we observed a significant skew to higher foci numbers. Chromosomes are segregated within the filaments, which is visible in cells stained with Hoechst 33342 (Fig. 3A) and as shown before [21]. In addition, in an overlay showing origins, termini and the Hoechst staining it becomes clear that per chromosome equivalent, the typical 2 origins and 1 terminus distribution can be visualised (Fig. 3C).

Similarly, for Cas1-Cas2 foci we observed an increase from a median number of 4 in the mock-treated control to 34 following the treatment with 40 µg/ml cephalixin for 60 min (Fig. 3D). Both systems show the same increase in heterogeneity observed with DnaN foci (Fig. 2B). However, as it is well established that replicated areas segregate continuously from ori to ter as replication proceeds [50–52], the problem of spatial resolution does not apply in the same way as for replisomes markers. The data therefore demonstrate that the observed increase is not simply caused by better spatial separation of foci but by a real increase in replication activity.

While we speculated that break-induced replication might contribute to the observed DNA synthesis in cells treated with saccharin [42], this seems unlikely following cephalixin treatment. Extensive generation of homologous recombination exchanges would result in physical links between sister chromatids, thereby preventing chromosome segregation [53]. However, as the chromosomes are segregated (Fig. 3A; 3C) [21], there is no indication of such links. In addition, upon removal of the drug, restart of division is observed following a lag, resulting in the formation of normal-sized cells that grow and divide without any further filamentation [40]. The observation that cells can resume division after cephalixin removal is inconsistent with extensive break-

induced replication as a major source of DNA synthesis. Large-scale BIR events would be expected to compromise genome integrity and lead to pronounced lethality, which was not observed under our experimental conditions.

This conclusion is further supported by our FROS analysis. In situations where DNA synthesis is blocked, such as following UV-induced DNA damage, continued *oriC* firing leads to amplification of origin-proximal regions, while terminus regions remain unreplicated and unsegregated until replication resumes [54]. Under these conditions, an increased number of origin foci is observed without a corresponding increase or segregation of terminus foci [40, 54, 55].

In contrast, following cephalixin treatment we observed increases in both origin and terminus foci, and terminus foci were distributed throughout elongated cells rather than remaining clustered (Fig. 3A). Although clusters of terminus foci were detected in some cells, most filaments displayed regularly spaced terminus signals, consistent with amplification and segregation of the terminus region. Furthermore, the average ori/ter ratio per cell was similar in mock- and cephalixin-treated cultures (Fig. 2B).

To rule out an involvement of DNA damage more directly, we analysed the formation of Cas1-Cas2 foci in cells following treatment with cephalixin in the presence or absence of the replication restart proteins PriB and PriC, respectively. We showed before that in *E. coli*, saccharin treatment results in an increase of DnaN-YPet foci, with a corresponding increase of Cas1-Cas2 foci [42]. By using the Cas1-Cas2 reporter system, we demonstrated that in cells lacking PriB and PriC, respectively, a significant reduction in the number of Cas1-Cas2 foci is observed in comparison to wild type cells [42]. We re-plotted the raw data for wild type and a $\Delta priB$ mutant following treatment with saccharin in Figure 4A in the same format of a Violin plot to allow a direct comparison. The reduced number of foci demonstrates that the high number of foci in saccharin treated cells requires in part the activity of the restart proteins PriB and PriC, suggesting that some form of DNA damage is generated. This DNA damage interferes with replisome progression,

which in turn requires synthesis to be re-established with the aid of the restart proteins. We hypothesised that if cephalexin is not inducing any DNA damage, the absence of PriB or PriC should not result in any reduction of Cas1-Cas2 foci.

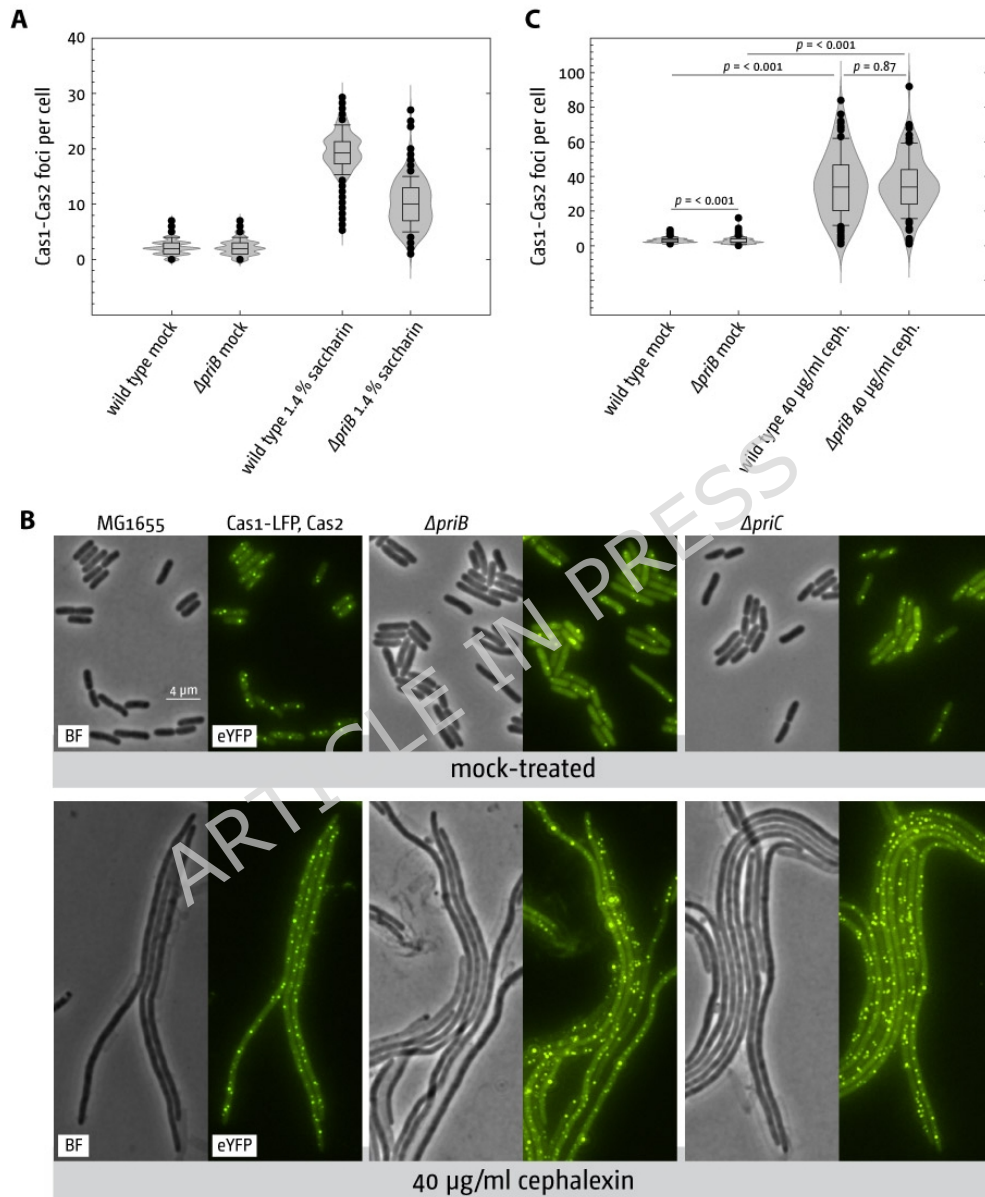


Figure 4. Cas1-LFP-Cas2 foci in replication restart mutants after cephalaxin treatment.

(A) Cas1-LFP-Cas2 foci in wild type and restart mutants. Cells were grown in LB (Miller) to $A_{600} = 0.2$ and mock-treated or treated with saccharin for 60 min while inducing Cas1-LFP-Cas2 expression with 0.1% arabinose [37]. Foci were counted from three independent experiments using ≥ 3 random frames per replicate, and data were pooled.

Numbers of cells analysed: 1312 (mock wild type), 560 (saccharin wild type), 795 (mock $\Delta priB$), and 274 (saccharin $\Delta priB$). Data were re-plotted from de Dios *et al.* [42] for comparison.

(B) Cells were grown as in (A) and mock-treated or treated with cephalixin for 60 min with simultaneous induction of Cas1-LFP-Cas2. Strains: JD1708 (wild type), RCe933 ($\Delta priB$), RCe934 ($\Delta priC$).

(C) Quantification of foci numbers for wild type and $\Delta priB$ cells from the experiment shown in (B). Counting was performed from two independent experiments using ≥ 3 random frames per replicate, and data were pooled. Wild type values were reproduced from Fig. 3 for comparison. Numbers of cells analysed: 625 (mock $\Delta priB$) and 87 (cephalexin $\Delta priB$). Quantification was focused on $\Delta priB$, which shows the stronger restart-dependent phenotype compared to $\Delta priC$ [42] and therefore represents the more stringent test for reduced synthesis.

This is precisely what we observed (Fig. 4B & 4C). Foci numbers remained high even in the absence of PriB and PriC, respectively. We therefore quantified Cas1-Cas2 foci in cells lacking PriB, which in our previous analysis showed the stronger reduction in foci numbers compared to a $\Delta priC$ mutant following saccharin treatment [42]. Because $\Delta priB$ represents the more sensitive background for detecting restart-dependent synthesis, this strain provides the more stringent test for a possible reduction in replication activity. For this reason, quantitative analysis was focused on the $\Delta priB$ mutant, while images of both $\Delta priB$ and $\Delta priC$ strains are shown for comparison, highlighting the lack of a difference (Fig. 4B). These data suggest that replication restart plays no role in the increased levels of DNA synthesis following cephalixin treatment. Even though this result does not fully rule out that some form of DNA damage may contribute towards the high levels of synthesis, it certainly rules out a number of specific mechanisms, including synthesis triggered at homologous recombination intermediates as well as break-induced replication (BIR) [56]. Taken together, our results suggest some form of acceleration of normal DNA replication is most likely to be responsible for the increased foci numbers observed.

Most synthesis in cephalixin-treated cells is DnaA-dependent

To investigate how much of the synthesis observed is triggered by origin firing, we used the temperature-sensitive *dnaA46* allele. If *dnaA46* cells are shifted to restrictive temperature, initiations at *oriC* are inhibited, while all ongoing rounds of DNA synthesis will proceed. When cells were grown at permissive temperature, more than 70% of cells had between 1 and 4 foci, with

only ~8% showing no foci at all (Fig. 5A & 5B), in line with most cells not only showing active DNA replication but also overlapping rounds of DNA synthesis. The numbers are slightly lower than shown in Fig. 2C, which is a temperature effect (30°C vs 37°C). Following shift to restrictive temperature for 90 min in mock-treated cells, 90% of cells showed 0 or 1 DnaN foci, with the fraction of cells with 0 foci increasing from 9% to almost 65% (Fig. 4A & 4B), in line with replication coming to an end while no additional rounds of synthesis can be initiated at *oriC*.

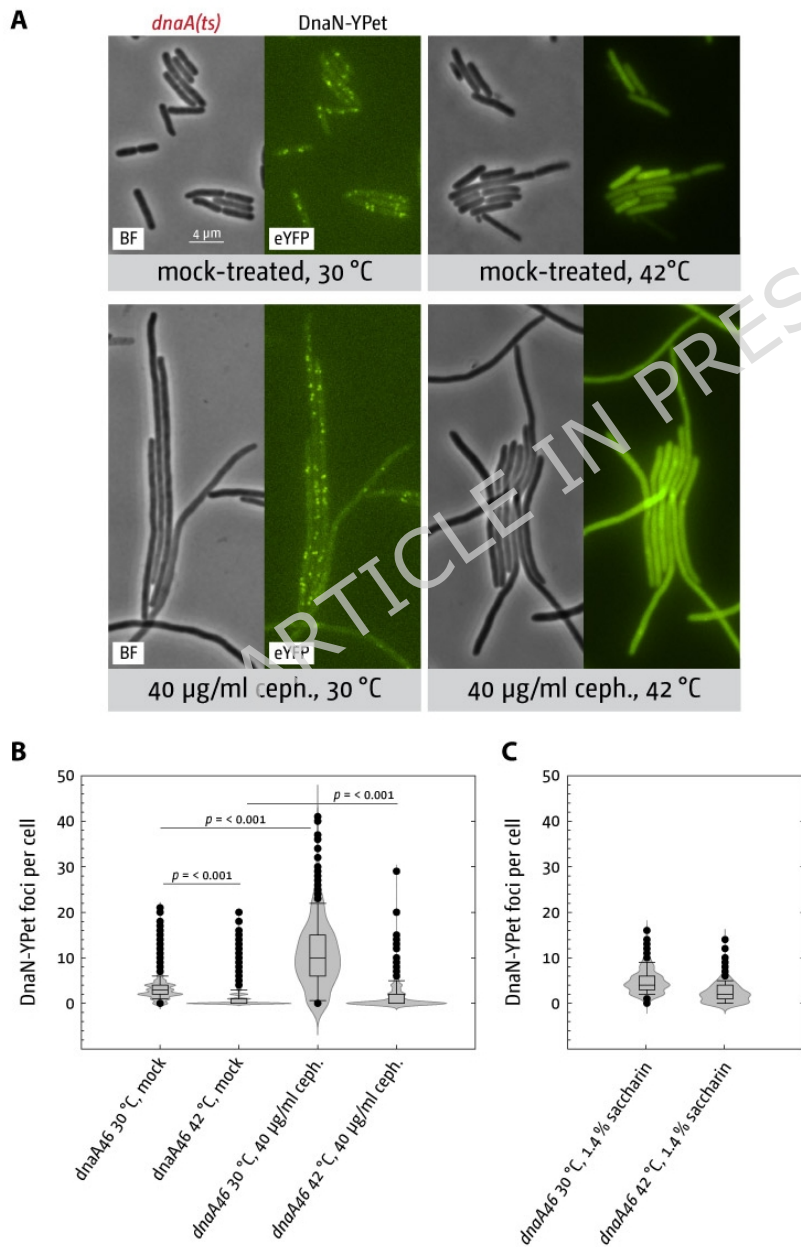


Figure 5. Effect of DnaA inactivation on replication activity in cephalixin-treated cells.

(A) Cells carrying the temperature-sensitive *dnaA46* allele (strain JD1460, *dnaA46 ypet-dnaN*) were grown in LB (Miller) at 30 °C to $A_{600} = 0.2$, split into four aliquots and incubated at 30 °C or 42 °C for 30 min before mock treatment or addition of 40 µg/ml cephalixin. Cultures were incubated for a further 60 min before imaging.

(B) Quantification of replisome foci for mock- and cephalixin-treated cells at 30 °C and 42 °C from the experiment shown in (A). Foci were counted from three independent experiments using ≥ 3 random frames per replicate, and data were pooled. Numbers of cells analysed: 995 (mock 30 °C), 1888 (mock 42 °C), 272 (cephalixin 30 °C), 488 (cephalixin 42 °C).

(C) Cells treated with saccharin under the same temperature conditions as in (A). Data were re-plotted from de Dios [42]. Numbers of cells analysed: 339 (30 °C) and 320 (42 °C).

Following treatment with 40 µg/ml cephalixin for 60 min at the permissive temperature of 30°C, a large increase in the number of foci was observed, with ~50% of cells showing between 8 and 20 DnaN foci (Fig. 5A & 5B). The increase is not as high as before (Fig. 2B) due to the lower temperature. However, for cells incubated at 42°C for 90 min, including treatment with cephalixin for 60 min, almost 60% had 0 foci, a very similar number to the number observed in untreated cells. 19% of cells showed a single focus, again similar to the 25% observed in untreated cells. Cells with 3–8 foci did show an increase from 10 to 19%. However, in comparison to the increase of foci numbers observed following cephalixin treatment at the permissive temperature of 30°C, this increase is mild (Fig. 4A & 4B). Importantly, the reduction in the number of foci at 42°C following cephalixin treatment is much stronger than the effect we observed following treatment with saccharin (Fig. 5C and [42]). Thus, the fact that the absence of the restart protein PriB does not reduce foci numbers following treatment with cephalixin, and that active DNA synthesis disappears in the majority of cells in *dnaA46* cells at restrictive temperature strongly suggests that the high levels of DNA synthesis observed after cephalixin treatment are mostly DnaA-dependent, while repair synthesis, such as break-induced replication following homologous recombination, contributes much less than following saccharin treatment. Thus, cephalixin-treated cells show an increase in DNA replication activity, the majority of which is DnaA-dependent, likely representing additional initiation events at *oriC*. However, the precise

molecular mechanism for the observed alterations in replication dynamics remains to be determined.

As these effects are all observed in cells treated with cephalixin only, we wanted to know to what extent the problems highlighted apply in cells treated with both cephalixin and rifampicin. Because rifampicin blocks transcription, it prevents the accumulation of newly synthesised DnaA required to reach the threshold for *oriC* firing. Thus, if the additional DNA synthesis observed in cephalixin-treated cells is driven by repeated DnaA-dependent initiation events at *oriC*, co-treatment with rifampicin should abolish this increase in replication activity.

As shown in Fig. 6A, treatment of cells with rifampicin only results in an increased variability of cell lengths. However, while the median cell length is identical to mock treated cells, the average cell length is mildly decreased (4.2 vs 3.6 μm), showing that a larger fraction of cells shows a reduction in cell length (Fig. 6A), in line with the microscopy images shown in Fig. 6B. If cells are treated with both rifampicin and cephalixin, the cells look far more normal than the filaments seen following treatment with cephalixin only (Fig. 6B & 6C). It is likely that for the extensive filamentation new protein synthesis is needed, which will be suppressed if transcription is blocked by rifampicin. However, there is a significant increase in average cell sizes in comparison to cells treated with rifampicin only (3.6 vs 4.5 μm), showing that some filamentation is still induced by the addition of cephalixin even if the generation of new proteins is blocked. However, the reduction in filamentation also results in a reduced lysis of cells, even though some lysed cells are still observed (Fig. 6C, white arrows).

As expected, the number of cells in cultures treated with rifampicin and cephalixin that show DnaN foci is also significantly reduced. We found that out of 709 cells analysed just under 20% (19.3%) showed foci, in line with the idea that the majority of synthesis is DnaA-dependent. Of the fraction of cells displaying foci, 45% had either 1 or 2 clearly defined foci, indicating perhaps that within the 60 min incubation period DNA synthesis had simply not quite completed. However, 55% showed either multiple foci or clusters of badly defined and often merging foci throughout the cell (Fig. 6B, white arrows).

Importantly, a similar effect was also observed in cells treated with rifampicin only (Fig. 6B, white arrows). Thus, despite transcriptional inhibition by rifampicin, pre-existing cellular components can support additional *oriC*-dependent initiation events in a fraction of cells, revealing that replication run-out conditions are not entirely uniform at the single-cell level.

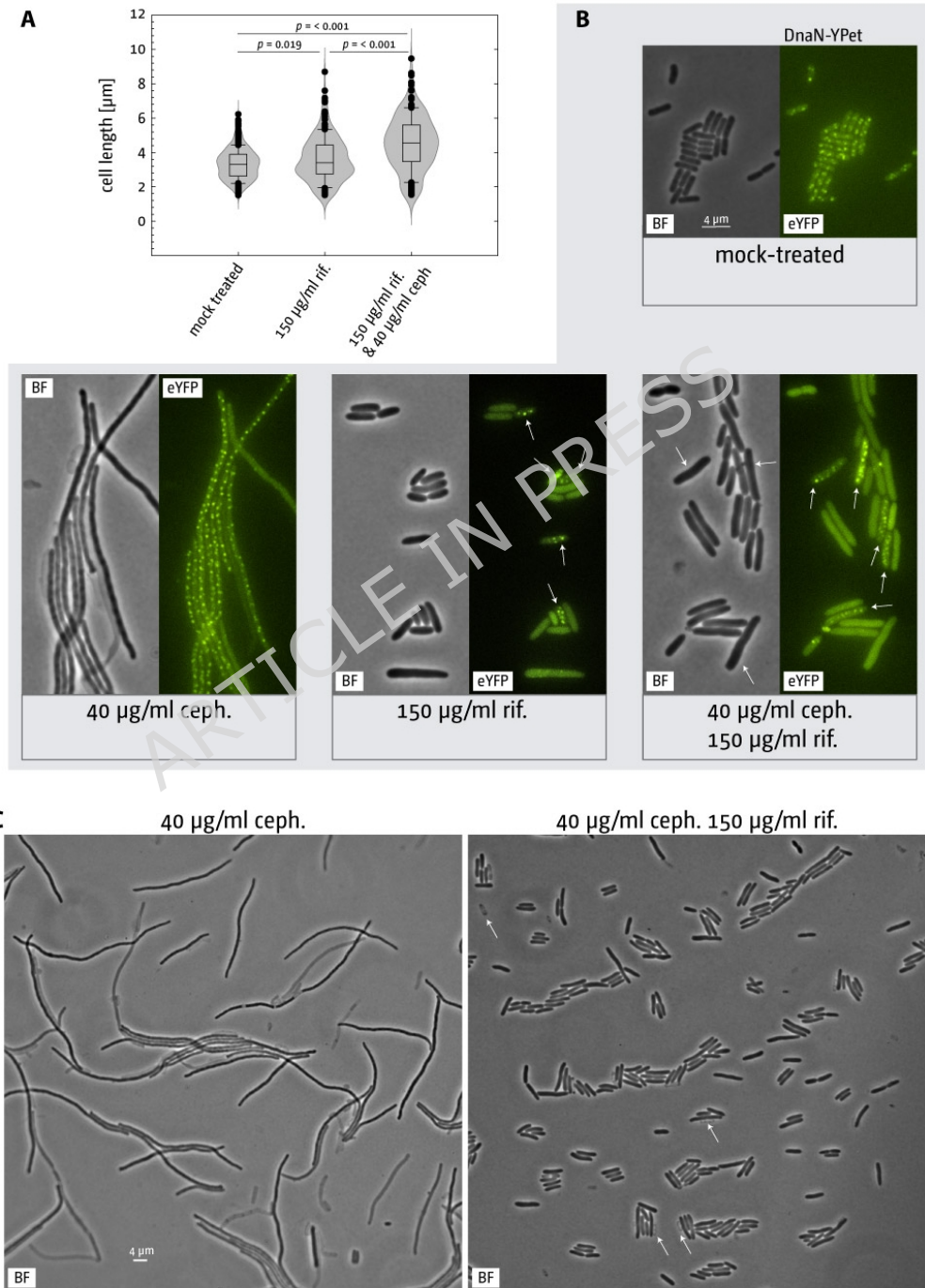


Figure 6. Replication dynamics in cells treated with cephallexin and rifampicin.

(A) Cell length analysis of cells mock-treated, treated with rifampicin, or treated with cephallexin plus rifampicin. Cells were grown in LB (Miller) to $A_{600} = 0.2$ and treated with the antibiotics indicated. Cephallexin-only lengths are not shown because extensive filamentation compresses the scale; these data are shown in Fig. 1B.

(B) Fluorescence micrographs of cells grown under the conditions shown in (A). Active replication complexes were visualised using YPet–DnaN (strain AS1062).

(C) Phase-contrast images of cells after 60 min treatment under the conditions shown in (A). Cephallexin causes strong filamentation and cell lysis, which is reduced when rifampicin is present, although some lysis events remain visible (white arrows). Entire fields are shown for comparison between conditions.

DISCUSSION

In this study we have investigated the effects that arise if *E. coli* cells are treated with the antibiotic cephallexin. Our data show that treatment with cephallexin alone results in rapid and significant filamentation of cells with formation of regular septal rings, as observed before [21, 32, 40]. In addition, cephallexin treatment results in an increased replication activity, as judged by replisome foci (YPet-DnaN; Fig. 2), Cas1-Cas2 foci as well as the direct visualisation of the numbers of origins and termini via FROS (Fig. 3). This increased replication activity is independent of the cephallexin concentration used (Fig. 2), and inactivation of the replication restart pathways ($\Delta priB$, $\Delta priC$) does not affect it in a major way (Fig. 4), a result that is in contrast to the situation observed before following treatment with saccharin [42]. Instead, inactivation of the replication initiator protein DnaA significantly reduces replication activity, highlighting that most of the synthesis observed following treatment with cephallexin is likely to be triggered at *oriC* (Fig. 5). Thus, our data establish a strong link between cephallexin treatment and DnaA-dependent replication activity. However, they do not directly address the molecular mechanism underlying this effect.

This line of experimentation was initiated in response to a reviewer who suggested for a different study to use cephallexin to block cell division and then study DNA replication and termination dynamics. The data presented here demonstrate that the use of cephallexin alone is not suitable to answer the question, because cephallexin treatment triggers a significant increase

in DNA replication activity. Thus, cephalixin changes DNA replication dynamics, which in turn will impact termination, possibly in unanticipated ways that are beyond what is observed in normally growing cells.

The fact that cephalixin triggers increased replication activity was surprising and triggered the question whether this effect has any impact on replication run-out experiments. It would be rather ironic if one of the drugs used to prevent cell division triggers more synthesis as an unwanted side effects, because this changes precisely one of the parameters this methodology is attempting to measure. If cephalixin were to have the same effect in replication run-out experiments, then the results would be artificially skewed towards higher numbers (Figs. 2–5).

To answer this question, we compared data from cells treated with the combination of rifampicin and cephalixin (Fig. 6) with the effects caused by cephalixin on its own (Figs. 1–5). Our data demonstrate that the increase in DNA replication activity is largely DnaA-dependent (Fig. 5), an effect that differs significantly from treatment with antimicrobial substances such as saccharin [42]. It is precisely the role of rifampicin to inhibit DnaA-dependent initiation, and our data show that it does so relatively effectively (Fig. 6).

However, we were surprised to find that in cultures treated with both rifampicin and cephalixin a fraction of cells remains that show an increase of YPet-DnaN foci (Fig. 6) – roughly 10% of all cells after treatment for 60 min. We suspected initially that this effect is triggered by cephalixin. However, the situation is not as simple, as a similar fraction of cells exists in cells treated with rifampicin only (Fig. 6). As rifampicin blocks transcription within a few minutes following its addition [57], this activity is likely to stem from the existing pool of proteins present in these cells. Consistent with this, DnaA has been shown to interact directly with RNA polymerase and to partially protect it from inhibition by rifampicin, which may allow limited transcription-dependent initiation to persist in a subset of cells even under run-out conditions [58]. The fraction of cells displaying elevated replication activity under run-out conditions appears small and is unlikely to substantially alter bulk DNA histograms in standard replication

run-out experiments. However, care should be taken when interpreting such data, particularly in sensitised mutant backgrounds where the proportion of responsive cells might differ from wild type and thereby skew comparative analyses. Moreover, approaches that rely on single-cell measurements – including high-resolution microscopy, microfluidics-based assays, or emerging single-cell transcriptomic and genomic techniques – would not average out this heterogeneity. Under such conditions, the presence of a replication-active subpopulation could disproportionately influence the interpretation of replication dynamics and cellular physiology.

In addition to the changes of DNA replication dynamics, the cell morphology changes triggered by cephalixin, including the extensive cell lysis, are also significant. The effect is obscured by the simultaneous treatment with rifampicin, reducing both filamentation and cell lysis, at least at the 60 min time point, but it is not completely alleviated (Fig. 6). Cells treated with both drugs can also be misshapen (Fig. 6), and at least some lysis of cells is still observed. Thus, the experimental outcome might change when mutants with changes in these or related metabolic pathways are analysed, or if any heterogeneity, for example by the presence of persister cells, is present. It would be good to give these and related aspects some consideration when designing replication run-out experiments.

The cephalixin concentrations most frequently used in experimental set-ups is

10 $\mu\text{g/ml}$, which is lower than what was used for the majority of the experiments in this study [46, 47, 59, 60]. However, as shown in Fig. 1 the level of synthesis visualised via DnaN foci is near identical for both 10 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$. In addition, higher concentrations have been used in a variety of studies [61–63]. One problem with replication run-out as an established methodology in this context is that the precise roles of the drugs used in these studies are not always clearly explained. Cephalixin is often introduced solely as a septation inhibitor. This is not entirely wrong, but our study demonstrates that this description clearly is incomplete. It would be important to state that cephalixin is a β -lactam antibiotic that can trigger cell lysis and, at low concentration, resistance of cells. It might be relevant to perform a systematic analysis of how

many cells are lysed over the duration of the run-out procedure, especially if multiple mutants are analysed.

Why is treatment with cephalixin triggering the observed increased levels of synthesis? Cephalixin specifically inhibits the transpeptidase activity of FtsI (PBP3) [41], and there is little indication that it would directly impact *oriC* activity. We suspect that the rather drastic increase in the number of replisomes (Fig. 2), which is accompanied with an increase of origin and termination areas (Fig. 3) as well as Cas1-Cas2 complexes (Fig. 3), is at least in part linked to the increased volume of cells treated with cephalixin (Fig. 1). Our mock-treated controls show 2–4 origin foci in most cells (Fig. 3). With a doubling time of ~20 min in our lab [11, 45] and a genome duplication time of ~40–50 min [12], 3 divisions would be expected to be the maximum. As the cultures grown are asynchronous, only a fraction of cells will be able to undergo 3 divisions. The majority of cells will undergo 2 divisions. We therefore would expect to see between 8 and 16 origins in most cells, with a fraction showing between 16 and 24. However, the median observed was 28 origins (Fig. 3), meaning that half the population showed foci numbers above 28. Taken together, the data clearly suggest that replication is initiated at the origin more than expected, in line with the increased numbers of replisomes (Fig. 2) and also the large numbers of Cas1-Cas2 complexes observed (Fig. 3). It was described before that there are direct links between the cell size and DnaA activity [64–66]. Although we did not directly quantify DnaA levels, we suspect that by drastically increasing cell size, cephalixin might trigger over-initiation at *oriC*, at least in some of the cells. Indeed, this might explain why the effect is so relatively independent of the cephalixin dose used (Fig. 2B), as the extent of the filamentation at 10 µg/ml and 40 µg/ml is very similar (Fig. 1B and 2A). While this model is consistent with current understanding of DnaA regulation and cell size control, experimental validation of this hypothesis will require dedicated mechanistic studies that are beyond the scope of the present work. Our aim here is not to redefine the molecular basis of this response, but to highlight its existence and potential impact on experimental interpretation.

These findings raise the question of whether alternative approaches might reduce some of the unintended physiological consequences associated with cephalixin treatment. With an array of new tools and procedures available, the necessity of drug treatment as part of the run-out method might be avoidable. Some questions can be easily solved using other experimental systems, such as FROS for the determination of the numbers of origins at a certain time. However, it has to be noted that other aspects are much more complicated to tackle. For example, replication run-out can be used to determine the fraction of cells in which chromosome duplication is unfinished due to replication fork stalling [67]. This can be determined using other methodologies, but these are neither as simple nor as relatively inexpensive as the replication run-out procedure. It might be interesting to investigate whether alternative drugs to cephalixin could be used to at least partly reduce the unwanted side effects of the current experimental procedure. However, the data shown in this study are a stark reminder of how much drug treatment can distort cellular morphology, and that care must be taken when using such procedures. Future studies will be required to determine how inhibition of cell division is mechanistically coupled to DnaA-dependent replication control under these conditions.

There are also clinical implications arising from this work. Antibiotic treatment can induce cellular stress responses that influence DNA replication and mutation rates, thereby contributing to bacterial evolvability [68, 69]. An increase in replication activity, as observed here following cephalixin treatment, may increase the opportunity for replication-associated mutations to arise. Notably, cephalixin alone induces extensive filamentation and cell lysis, whereas these effects are substantially reduced when cephalixin is combined with rifampicin, indicating a physiological antagonism between the two drugs under these conditions. While these observations are made in a laboratory context, they highlight the importance of considering how antibiotic-induced physiological responses – including antagonistic interactions in combination treatments – can influence replication dynamics, cell viability, and ultimately bacterial survival.

CONCLUSION

Here we show that cephalixin treatment induces substantial and reproducible changes in cellular physiology and DNA replication dynamics in *E. coli*, including DnaA-dependent increases in DNA synthesis and extensive cell filamentation and lysis. These effects occur under similar conditions used in experimental and clinical contexts and are therefore relevant for the interpretation of antibiotic-induced phenotypes more broadly.

Our data show that, while rifampicin reduces these effects, it does not fully suppress aberrant replication activity in all cells, highlighting a degree of heterogeneity that may influence experimental outcomes. While this is likely to be less problematic in population experiments, it will become important in certain mutant backgrounds and single-cell analyses in particular.

Our data do not allow us to resolve whether the observed increase in DNA synthesis is a direct consequence of cephalixin treatment or arises indirectly from the inhibition of cell division. Nevertheless, our findings define a robust physiological phenotype and establish clear constraints that should be considered when interpreting antibiotic-based assays, including replication run-out experiments.

Taken together, this work emphasises the need for careful evaluation of antibiotic-based experimental systems and provides a framework for interpreting replication dynamics and cellular responses in their presence.

DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and analysed during the current study are available via the Brunel Figshare repository: <https://doi.org/10.17633/rd.brunel.31999347>

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

IG [designed/performed experiments; analysed data; contributed to editing]. DK [performed experiments; contributed reagents; analysed data; contributed to editing]. ELD [performed experiments; analysed data; contributed to editing]. SP [performed experiments; analysed data]. RRM [conceptualised the study; supervised the project; acquired funding; contributed to editing]. CJR [conceptualised the study; supervised the project; acquired funding; wrote the first draft of the manuscript; contributed to editing]. All authors read and approved the final manuscript.

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TABLES

Table 1. *Escherichia coli* K12 strains

Strain number	Relevant Genotype ^a	Source
AB1157 derivatives		
RRL189	<i>lacO240::hyg</i> at <i>ori1</i> ^b , <i>tetO240::gen</i> at <i>ter3</i> ^b , <i>P_{lac}-lacI-mCherry-frt</i> at <i>leuB</i> , <i>P_{lac}-tetR-</i> <i>mCerulean-frt</i> at <i>galK</i>	[70]
RRL190	< <i>kan</i> >- <i>ypet-dnaN</i>	[71]
RRL309	<i>fis-mCherry-<cat></i> pCP8 ^d	David Sherratt
MG1655 derivatives		
MG1655	F ⁻ rph-1	[72]
AM1784	<i>ΔpriC::dhfr</i>	A.A. Mahdi & R.G. Lloyd, unpublished
AM2013	<i>ΔpriB::dhfr</i>	[73]
AM2017	<i>ΔlacIZYA ΔpriB::dhfr</i>	TB28 × P1.AM2013 to Tm ^r
AU1054	<i>tnaA::Tn10 dnaA46</i>	[74]
AS1062	< <i>kan</i> >- <i>ypet-dnaN</i>	MG1655 × P1.RRL190 to Km ^r

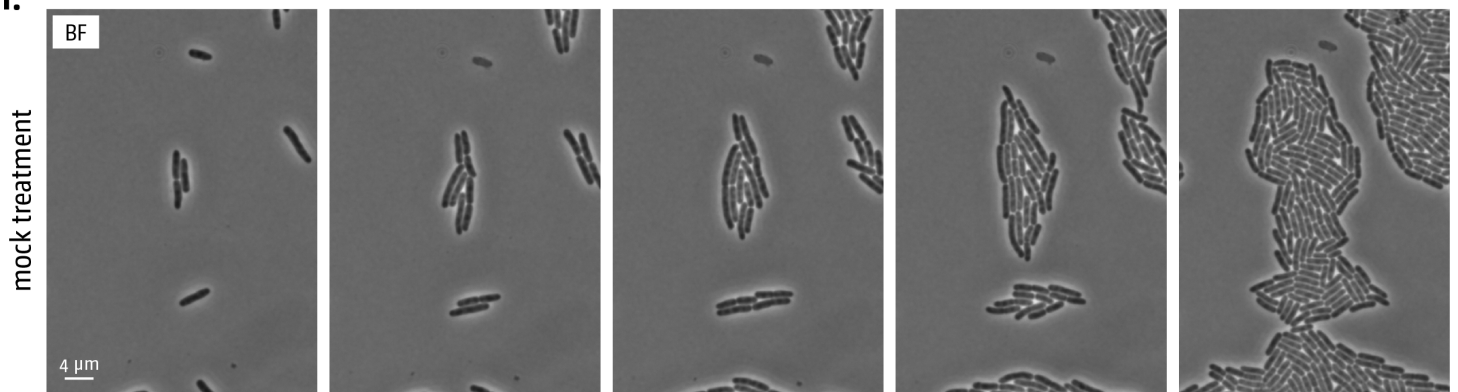
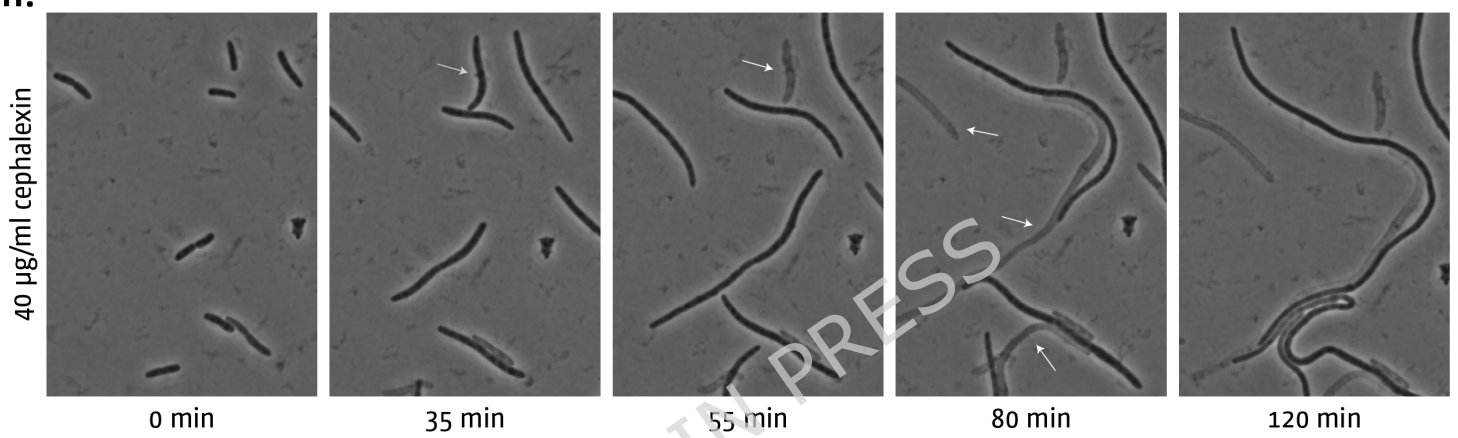
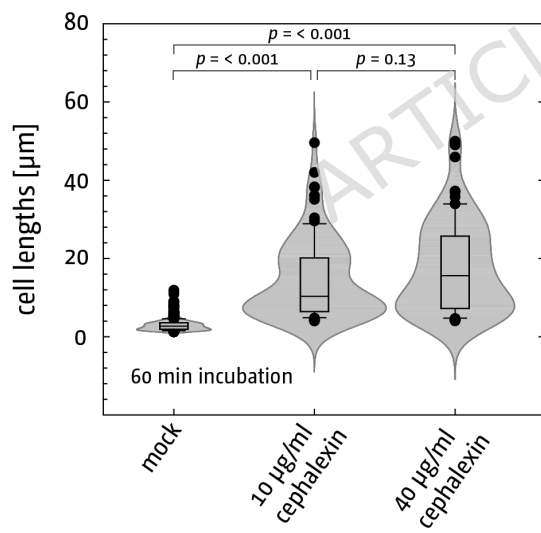
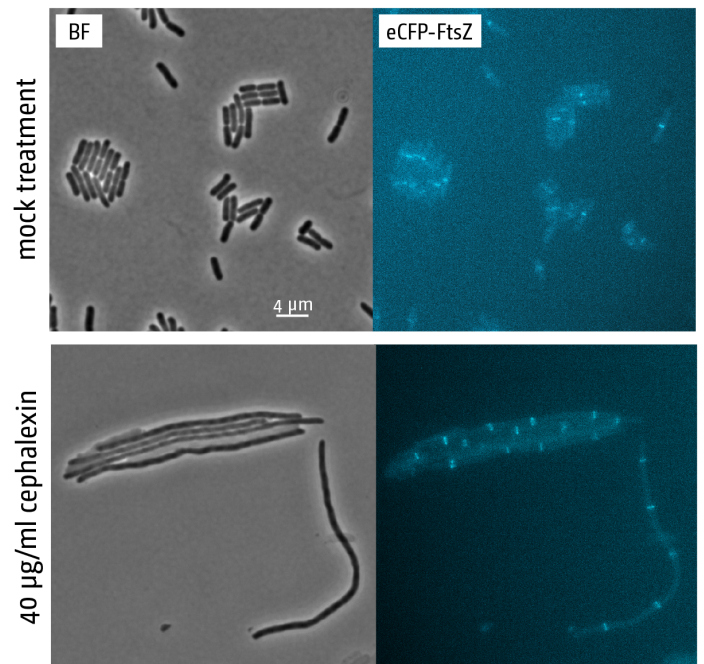
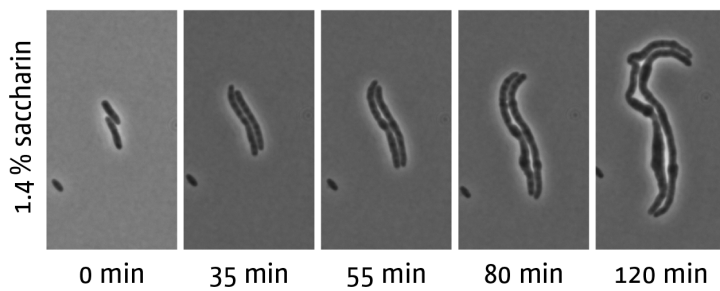
JD1460	<i>tnaA::Tn10 dnaA46 <kan>-ypet-dnaN</i>	This study
JD1669	<i>ΔpriB::dhfr</i>	MG1655 × P1.AM2017 to Tm ^r
JD1708	MG1655 pTK135 ^c	[37]
JD1709	<i>fis-mCherry-<cat></i>	MG1655 × P1.RRL309 to Cm ^r
JD1853	<i>ΔpriC::dhfr</i>	MG1655 × P1.AM1784 to Tm ^r
JD1841	<i>lacO240::hyg</i> at <i>ori</i> ^b , <i>tetO240::gen</i> at <i>ter3</i> ^b , <i>P_{lac}-lacI-mCherry-frt</i> at <i>leuB</i> , <i>P_{lac}-tetR-mCerulean-frt</i> at <i>galK</i> pTK135	RRL189 × pTK135 to Ap ^r
RCe849	<i>fis-mCherry-<cat></i> pCP8 ^d	JD1709 × pCP8 to Ap ^r
RCe933	<i>ΔpriB::dhfr</i> pTK135 ^c	JD1669 × pTK135 to Ap ^r
RCe934	<i>ΔpriC::dhfr</i> pTK135 ^c	JD1853 × pTK135 to Ap ^r
TB28	<i>ΔlacIZYA</i>	[75]

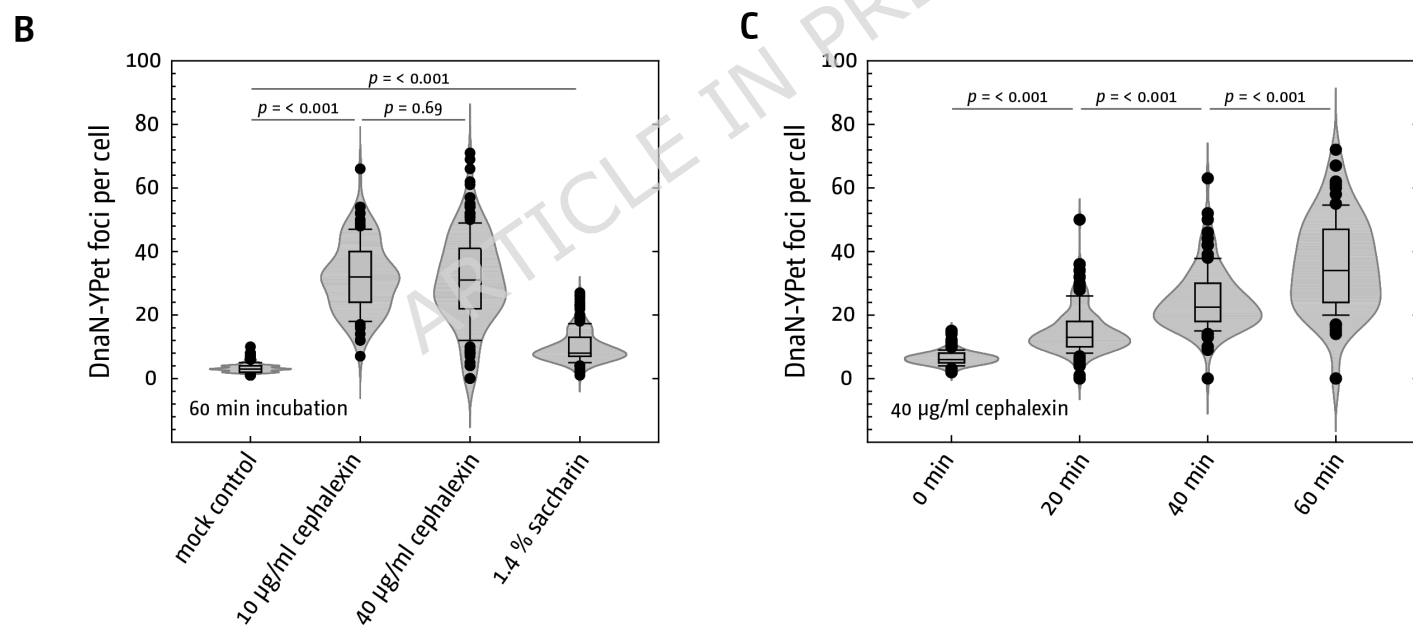
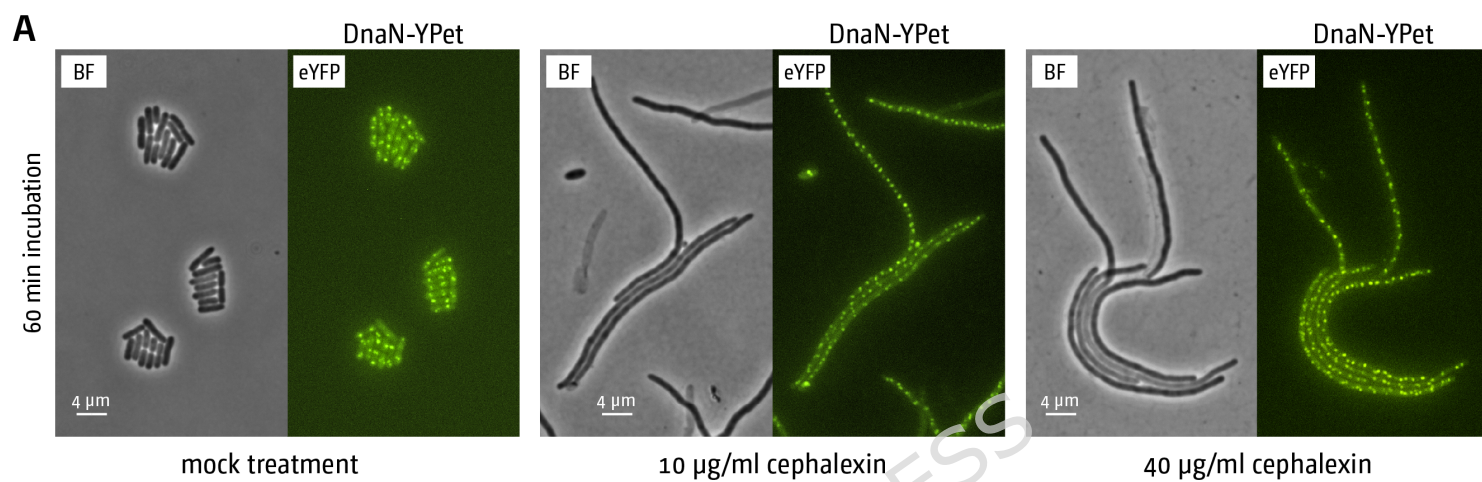
a – Only the relevant additional genotype of the derivatives is shown. The abbreviations ‘dhfr’, ‘Tn10’, ‘kan’, ‘hyg’, ‘gen’, and ‘cat’ refer to insertions conferring resistance trimethoprim (Tm^r), tetracyclin (Tc^r), kanamycin (Km^r), hygromycin (Hyg^r), gentamicin (Gen^r), and chloramphenicol (Cm^r). ‘<>’ indicates the use of *frt* sites, where *frt* stands for the 34 bp recognition site of the FLP/*frt* site-directed recombination system. Thus, <cat> refers to a chloramphenicol marker flanked by an *frt* site either side.

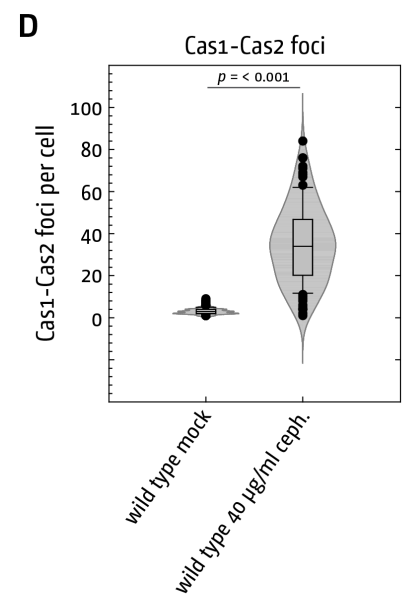
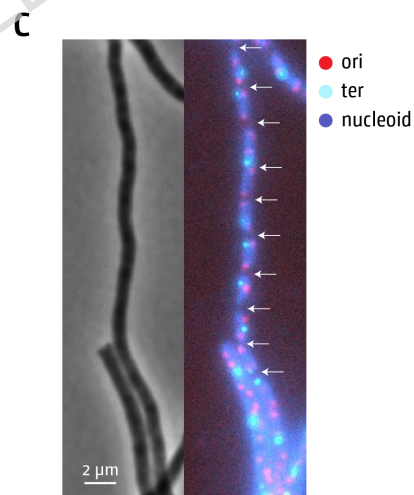
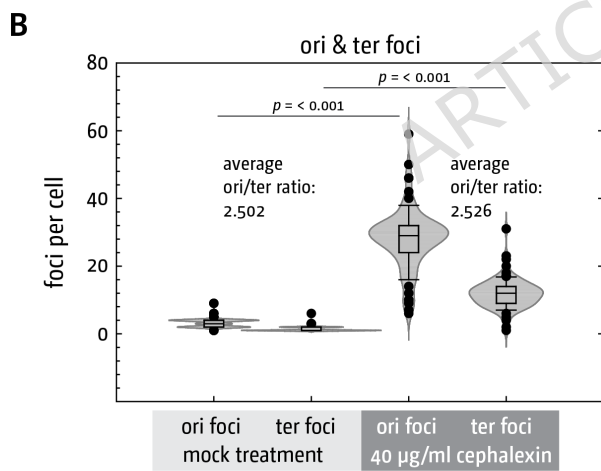
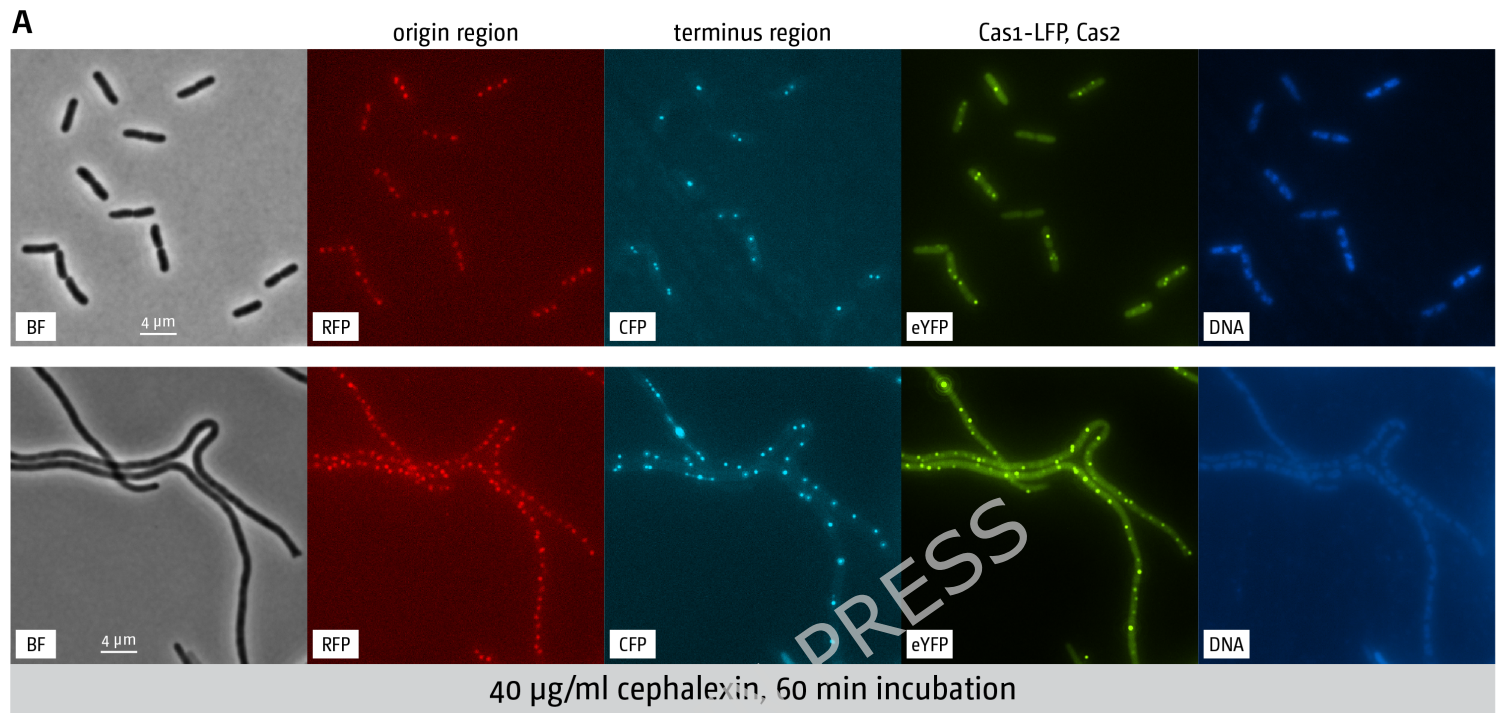
b – *ori1* is located 16 kb anticlockwise of *oriC*; *ter3* is located 50 kb clockwise from *dif*.

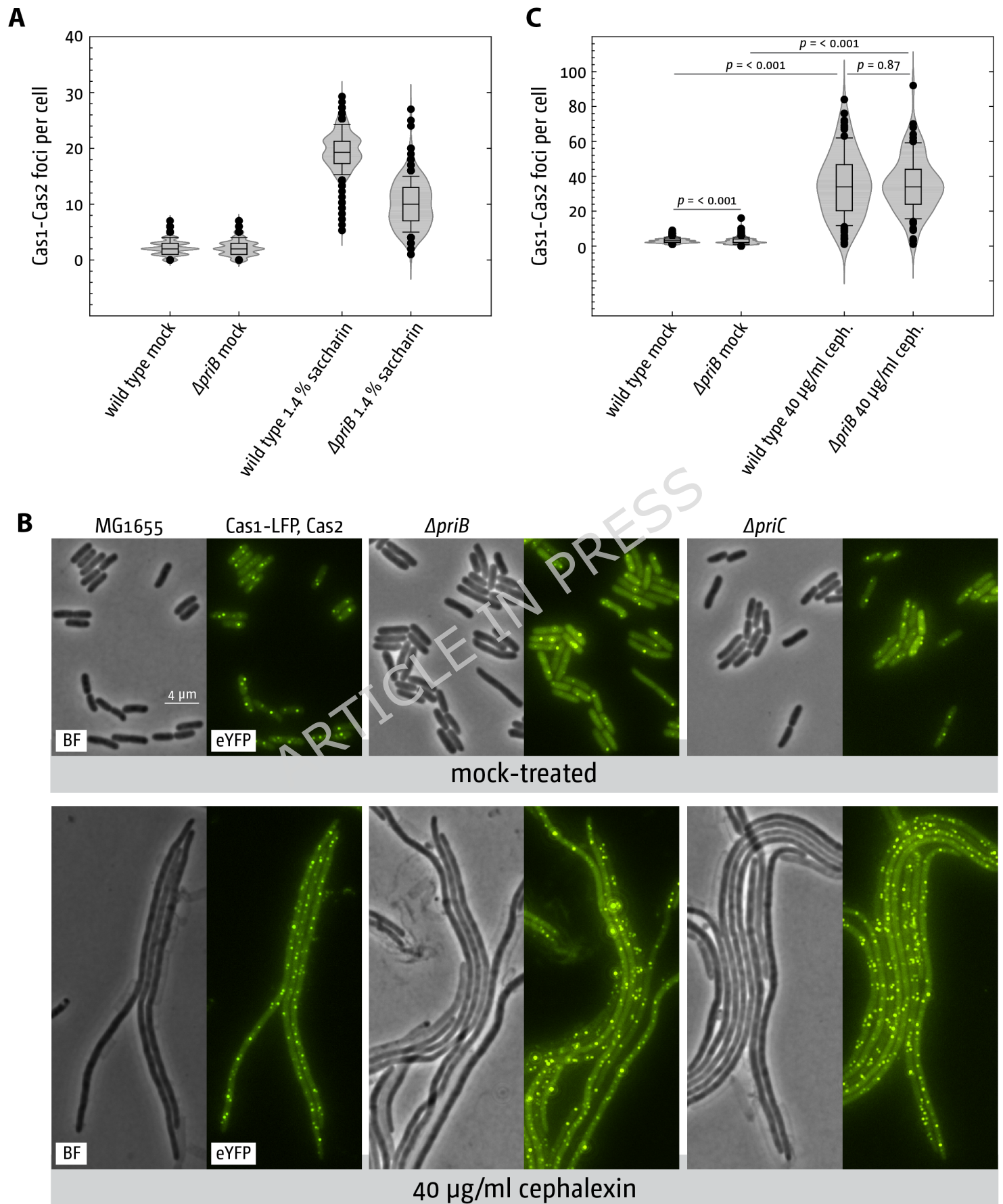
c – For details about the Cas1-LFP-Cas2 expression plasmid pTK135 see [37].

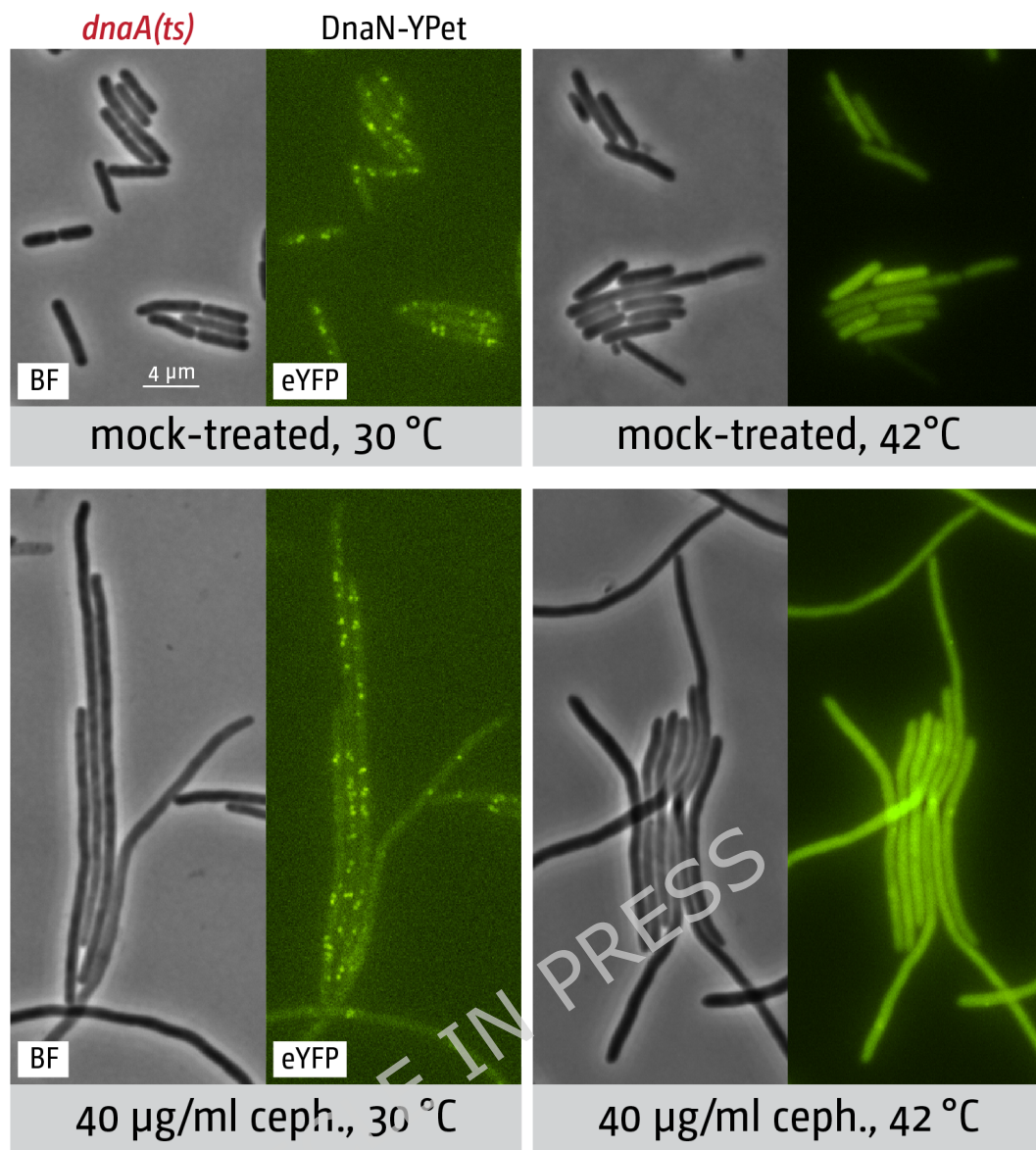
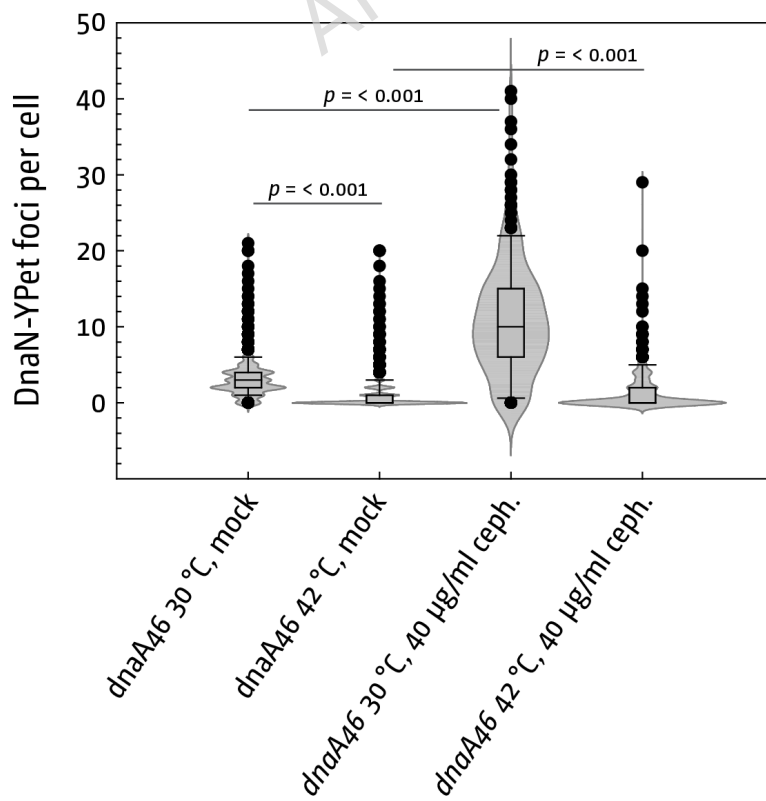
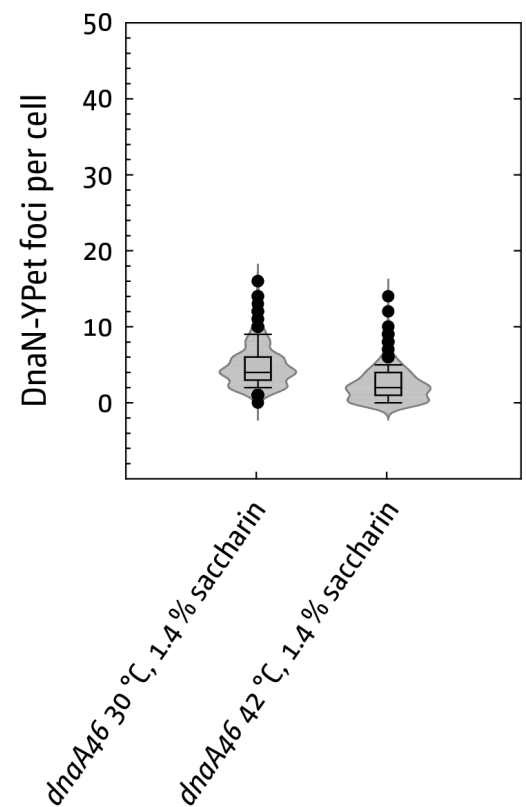
d – pCP8 is a multicopy pBR322 derivative (Ap^r) carrying *pftsKi-ftsZ-cfp*. The plasmid allows the constitutive low expression of a FtsZ-CFP fusion protein from a weak promoter within FtsK [76].

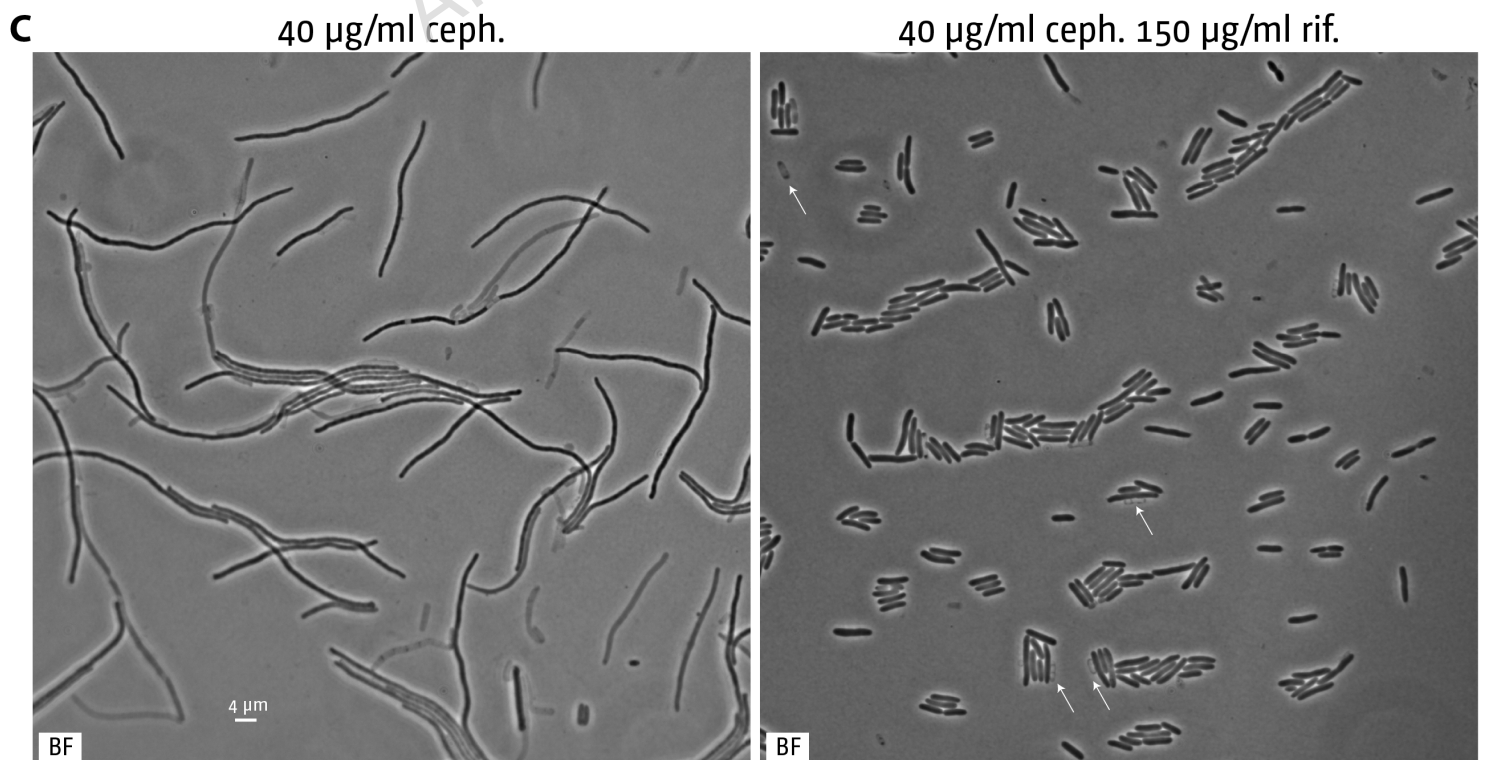
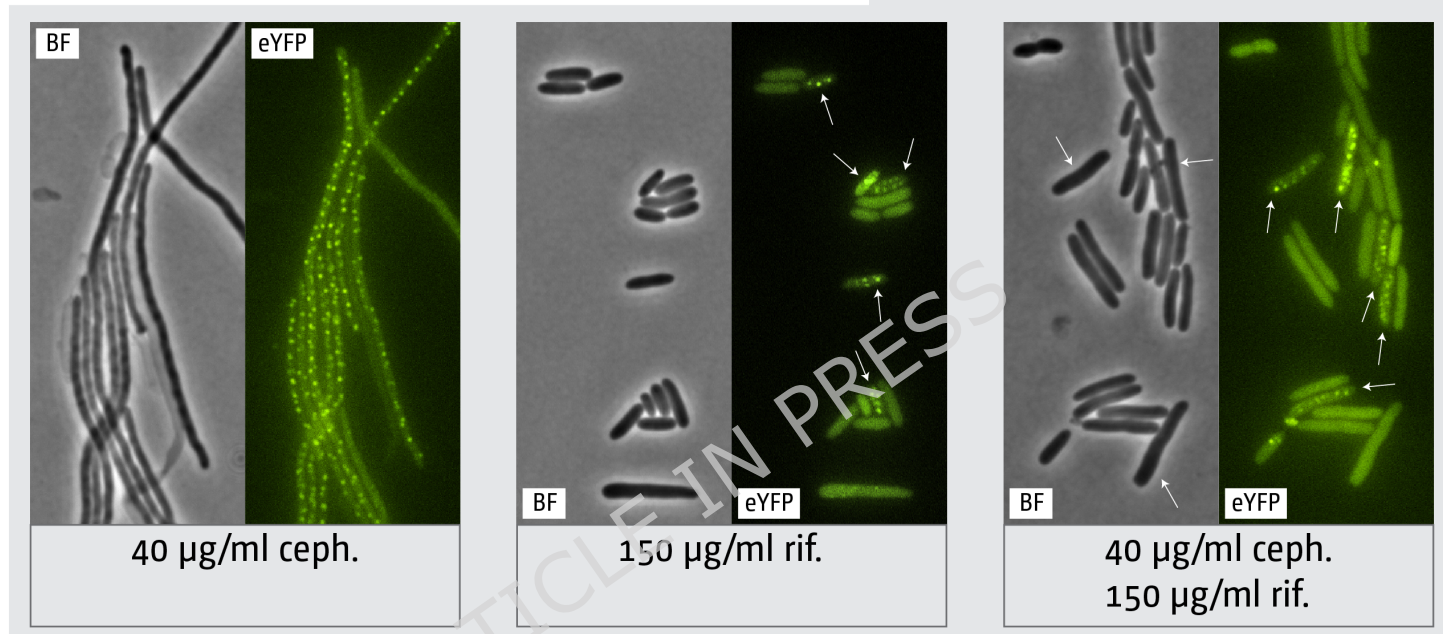
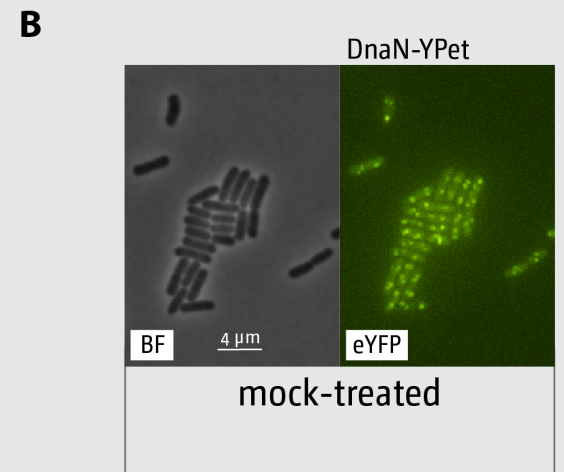
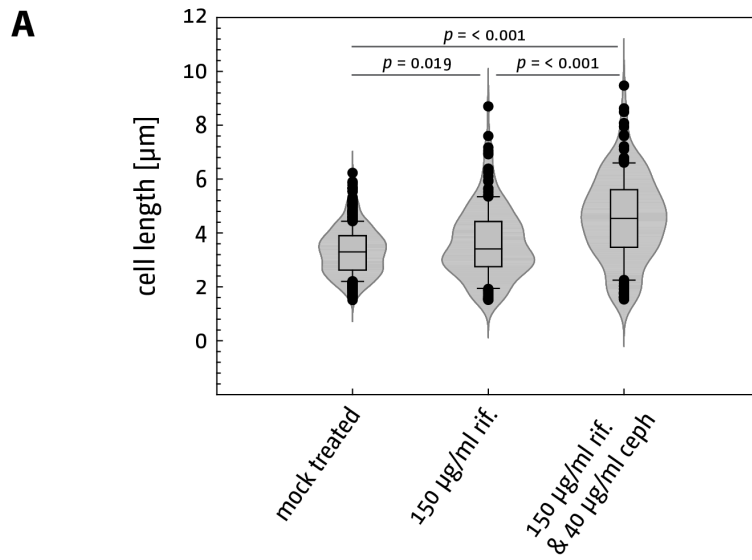
A I.**A II.****B****C****D**







A**B****C**



TABLES

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Strain number	Relevant Genotype ^a	Source
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RRL190	< <i>kan</i> >- <i>ypet-dnaN</i>	[71]
RRL309	<i>fis-mCherry-<cat></i> pCP8 ^d	David Sherratt
MG1655 derivatives		
MG1655	F ⁻ <i>rph-1</i>	[72]
AM1784	<i>ΔpriC::dhfr</i>	A.A. Mahdi & R.G. Lloyd, unpublished
AM2013	<i>ΔpriB::dhfr</i>	[73]
AM2017	<i>ΔlacIZYA ΔpriB::dhfr</i>	TB28 × P1.AM2013 to Tm ^r
AU1054	<i>tnaA::Tn10 dnaA46</i>	[74]
AS1062	< <i>kan</i> >- <i>ypet-dnaN</i>	MG1655 × P1.RRL190 to Km ^r
JD1460	<i>tnaA::Tn10 dnaA46 <kan>-ypet-dnaN</i>	This study
JD1669	<i>ΔpriB::dhfr</i>	MG1655 × P1.AM2017 to Tm ^r
JD1708	MG1655 pTK135 ^c	[37]
JD1709	<i>fis-mCherry-<cat></i>	MG1655 × P1.RRL309 to Cm ^r
JD1853	<i>ΔpriC::dhfr</i>	MG1655 × P1.AM1784 to Tm ^r
JD1841	<i>lacO240::hyg</i> at <i>ori1</i> ^b , <i>tetO240::gen</i> at <i>ter3</i> ^b , <i>P_{lac}-lacI-mCherry-frt</i> at <i>leuB</i> , <i>P_{lac}-tetR-</i> <i>mCerulean-frt</i> at <i>galK</i> pTK135	RRL189 × pTK135 to Ap ^r
RCe849	<i>fis-mCherry-<cat></i> pCP8 ^d	JD1709 × pCP8 to Ap ^r
RCe933	<i>ΔpriB::dhfr</i> pTK135 ^c	JD1669 × pTK135 to Ap ^r
RCe934	<i>ΔpriC::dhfr</i> pTK135 ^c	JD1853 × pTK135 to Ap ^r
TB28	<i>ΔlacIZYA</i>	[75]

a – Only the relevant additional genotype of the derivatives is shown. The abbreviations ‘dhfr’, ‘Tn10’, ‘kan’, ‘hyg’, ‘gen’, and ‘cat’ refer to insertions conferring resistance trimethoprim (Tm^r), tetracyclin (Tc^r), kanamycin (Km^r), hygromycin (Hyg^r), gentamicin (Gen^r), and chloramphenicol (Cm^r). ‘<>’ indicates the use of *frt* sites, where *frt* stands for the 34 bp recognition site of the FLP/*frt* site-directed recombination system. Thus, <*cat*> refers to a chloramphenicol marker flanked by an *frt* site either side.

b – *ori1* is located 16 kb anticlockwise of *oriC*; *ter3* is located 50 kb clockwise from *dif*.

c – For details about the Cas1-LFP-Cas2 expression plasmid pTK135 see [37].

d – pCP8 is a multicopy pBR322 derivative (Ap^r) carrying *pftsKi-fsZ-cfp*. The plasmid allows the constitutive low expression of a FtsZ-CFP fusion protein from a weak promoter within FtsK [76].