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Antibiotic-Induced Replication Stress Triggers Bacterial Competence by Increasing Gene Dosage near the Origin

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SUMMARY

Streptococcus pneumoniae (pneumococcus) kills nearly 1 million children annually, and the emergence of antibiotic-resistant strains poses a serious threat to human health. Because pneumococci can take up DNA from their environment by a process called competence, genes associated with antibiotic resistance can rapidly spread. Remarkably, competence is activated in response to several antibiotics. Here, we demonstrate that antibiotics targeting DNA replication cause an increase in the copy number of genes proximal to the origin of replication (*oriC*). As the genes required for competence initiation are located near *oriC*, competence is thereby activated. Transcriptome analyses show that antibiotics targeting DNA replication also upregulate origin-proximal gene expression in other bacteria. This mechanism is a direct, intrinsic consequence of replication fork stalling. Our data suggest that evolution has conserved the *oriC*-proximal location of important genes in bacteria to allow for a robust response to replication stress without the need for complex gene-regulatory pathways.

INTRODUCTION

The pneumococcus is a Gram-positive human commensal, usually residing in the nasopharynx. Although mostly harmless, *Streptococcus pneumoniae* is able to cause invasive (pneumonia, septicemia, and meningitis) as well as noninvasive (otitis media, sinusitis, and bronchitis) diseases, particularly in children, the elderly, and the immunocompromised (O'Brien et al., 2009; Simell et al., 2012; Weiser, 2010). Thus, it is essential to get as full an understanding as possible of this bacterium to be able to effectively combat pneumococcal infections. Here, we focus on the ability of certain antibiotics to promote competence in *S. pneumoniae* (Prudhomme et al., 2006). In the competent state, cells are able to take up exogenous DNA, potentially leading to incorporation of new genes (e.g., providing antibiotic resistance) (Cornick and Bentley, 2012). Besides the cells being able to genetically transform themselves, multiple other functionalities are activated concomitantly, including DNA repair and bacteriocin production, thereby increasing their survival rate during stress (Claverys et al., 2006). For instance, survival of competent pneumococcal cells is significantly increased when chromosomal DNA is present and used for transformation during treatment with the DNA-damaging agent mitomycin C (MMC) (Engelmoer and Rozen, 2011). Induction of competence also increases the survival of cells treated with protein synthesis inhibitors kanamycin and streptomycin, but in that case actual transformation is not required (Engelmoer and Rozen, 2011). Instead, some other, unknown genes from the large competence regulon may be responsible for coping with perturbed protein synthesis. These characteristics suggest that competence may function as a general stress response, especially because *S. pneumoniae* lacks the SOS response present in many other bacteria (Charpentier et al., 2012).

The key proteins responsible for activating competence are ComABCDE and ComX, all of which are encoded by early *com* operons (*comAB*, *comCDE*, and *comX*) (Figure 1). ComAB, a membrane transporter, exports ComC, a small leader-containing peptide. The leader is cleaved off upon export, yielding the competence-stimulating peptide (CSP) (Håvarstein et al., 1995). ComDE functions as a two-component regulatory system as extracellular CSP interacts with the membrane-bound histidine kinase ComD, which subsequently activates ComE by phosphorylation (Martin et al., 2013). ComE∼P enhances expression of, among others, *comAB*, *comCDE*, and *comX*. The alternative sigma factor ComX (σ_X) then activates the late *com* genes, resulting in production of proteins required for DNA repair and transformation (Figure 1). When a certain threshold concentration of extracellular CSP is reached, cells get into an autocatalytic loop via transcriptional activation by ComE-P and ComC export, effectively switching on competence throughout the population (Charpentier et al., 2012; Claverys et al., 2006; Johnsborg and Håvarstein, 2009). Importantly, in noncompetent cells, the basal expression of *comCDE* depends on transcriptional read-through from the upstream $tRNA^{Arg5}$. Martin et al. proposed that this read-through is essential for a homogeneous, population-wide switch to the competent state (Martin et al., 2010). All in all, this regulatory network constitutes a sensitive switch, and even a slight imbalance in the system, for example the presence of an extra copy of *comC*,

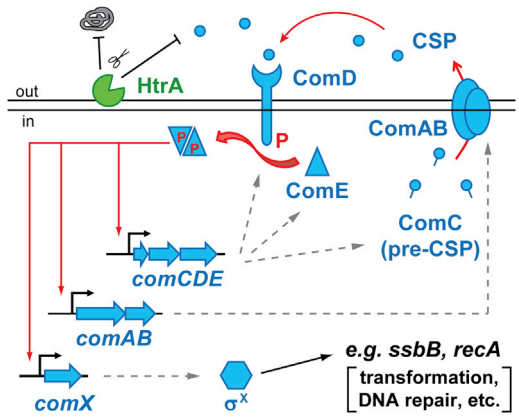

Figure 1. Overview of the Regulatory Network Driving Competence in *S. pneumoniae*

is enough to trigger the cascade (Alloing et al., 1998; Pestova et al., 1996). Whether or not cells will become naturally competent also depends on experimental conditions. Tomasz and Mosser, for example, showed in 1966 that transformation yields are strongly influenced by pH; at high pH (~8.0), competence occurs in early-exponential phase. If, however, initial pH is lowered, natural competence is delayed and weaker or completely absent (Tomasz and Mosser, 1966). In our experimental conditions, the highest pH at which no natural competence is observed is 7.4.

The emergence of *S. pneumoniae* strains with resistance to antibiotics poses a serious threat to human health. Because of the ability of *S. pneumoniae* to take up DNA from its environment by competence, genes associated with drug resistance rapidly spread, causing *S. pneumoniae* to develop into multidrug-resistant "superbugs" (Croucher et al., 2011; Simell et al., 2012). Frighteningly, Prudhomme et al. demonstrated that competence in *S. pneumoniae* is activated in response to several antibiotics (Prudhomme et al., 2006). Thus, inappropriate antibiotic treatments can accelerate the occurrence of multidrug resistance and promote the evolution of virulence. The mechanisms underlying antibiotic-induced competence in *S. pneumoniae* remain poorly understood. While antibiotics in general can cause global transcriptional responses in bacteria and activate general stress responses such as the SOS response, many other genes outside these regulons are also frequently differentially expressed (Wecke and Mascher, 2011). Here, we discover the existence of a general molecular mechanism that allows bacteria to challenge antibiotic-induced replication stress. We found that all antibiotics targeting DNA replication in bacteria (we tested *S. pneumoniae*, *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus*) cause stalled replication forks, while DNA replication initiation continues. This results in an increase in copy numbers of genes close to the origin of replication and subsequent global changes in transcription. In the case of *S. pneumoniae*, we show that this shifted gene-dosage results in activation of the competence pathway, which thereby allows the bacterium to take up foreign DNA and potentially acquire antibiotic-resistance genes.

RESULTS

DNA Replication Stress Activates Competence

Strikingly, certain antibiotics induce competence (Prudhomme et al., 2006). For instance, kanamycin and streptomycin are thought to promote competence by inducing decoding errors during translation, which leads to accumulation of misfolded proteins (Stevens et al., 2011). Because CSP and misfolded proteins are both targets of the HtrA protease (Cassone et al., 2012), it was speculated that the accumulation of misfolded proteins may occupy HtrA, thus derepressing competence (Stevens et al., 2011) (Figure 1). However, this HtrA-mediated mechanism cannot explain the activation of competence by MMC, a DNA-damaging agent, and by the topoisomerase poisons norfloxacin, levofloxacin, and moxifloxacin (Prudhomme et al., 2006). The *comCDE* operon is located within 3 kb of *oriC* (Table S1 available online), and it has been postulated that this colocalization might provide a way to regulate competence with DNA replication (Claverys et al., 2000). We wondered whether antibiotics affecting DNA replication in general would induce competence. To test this, we treated pneumococcal D39 cells (Avery et al., 1944) with 6-(p-hydroxyphenylazo)-uracil (HPUra). This drug is converted intracellularly to a deoxyguanosine triphosphate analog that reversibly binds DNA polymerase type III (PolC), thereby temporarily stalling the replication fork (Brown, 1970) and inducing replication stress (Figure 2A). It is important to note that the concentration of HPUra used (0.15 μg/ml) does not block DNA replication completely but, rather, slows down replication elongation. To monitor competence development, the firefly *luc* gene was inserted downstream of the late σ_x-dependent competence gene *ssbB*. Activation and expression of *ssbB* is a good indicator for actual transformation with externally added DNA, because cells expressing *ssbB-luc* are also competent for transformation (Prudhomme et al., 2006). When *S. pneumoniae* was grown in C+Y medium at a pH of 7.4 (which does not allow natural competence under our experimental conditions) and in the presence of 0.15 μg/ml HPUra, a clear growth defect was observed (Figure 2C). Importantly, under these conditions, competence was activated by HPUra-induced replication stress (Figure 2C). In line with the known genetic program driving competence development, transcription of *comCDE* was also activated by HPUra, which occurred slightly before activation of *ssbB* (Figure S1). Single-cell analysis showed that approximately 50% of the cells became competent upon HPUra treatment and robustly expressed green fluorescent protein (GFP) from the *ssbB* promoter (Figure 3A). The noncompetent fraction was likely nonviable, because the competent fraction did not increase further in the presence of added CSP and many cells were anucleate (Figure 3B). Crucially, cells exposed to HPUra were competent for actual transformation and readily took up and integrated exogenously added DNA containing a rifampicin resistance allele (a PCR product containing $rpoB^{D489V}$) and became resistant to this antibiotic (Figure 3C). Note that the transformation efficiency was significantly lower than 100% (Figure 3C), which can be explained by the fact that transformation is a rather stochastic process that depends on several factors, such as the DNA concentration, the nature of the DNA (e.g.,

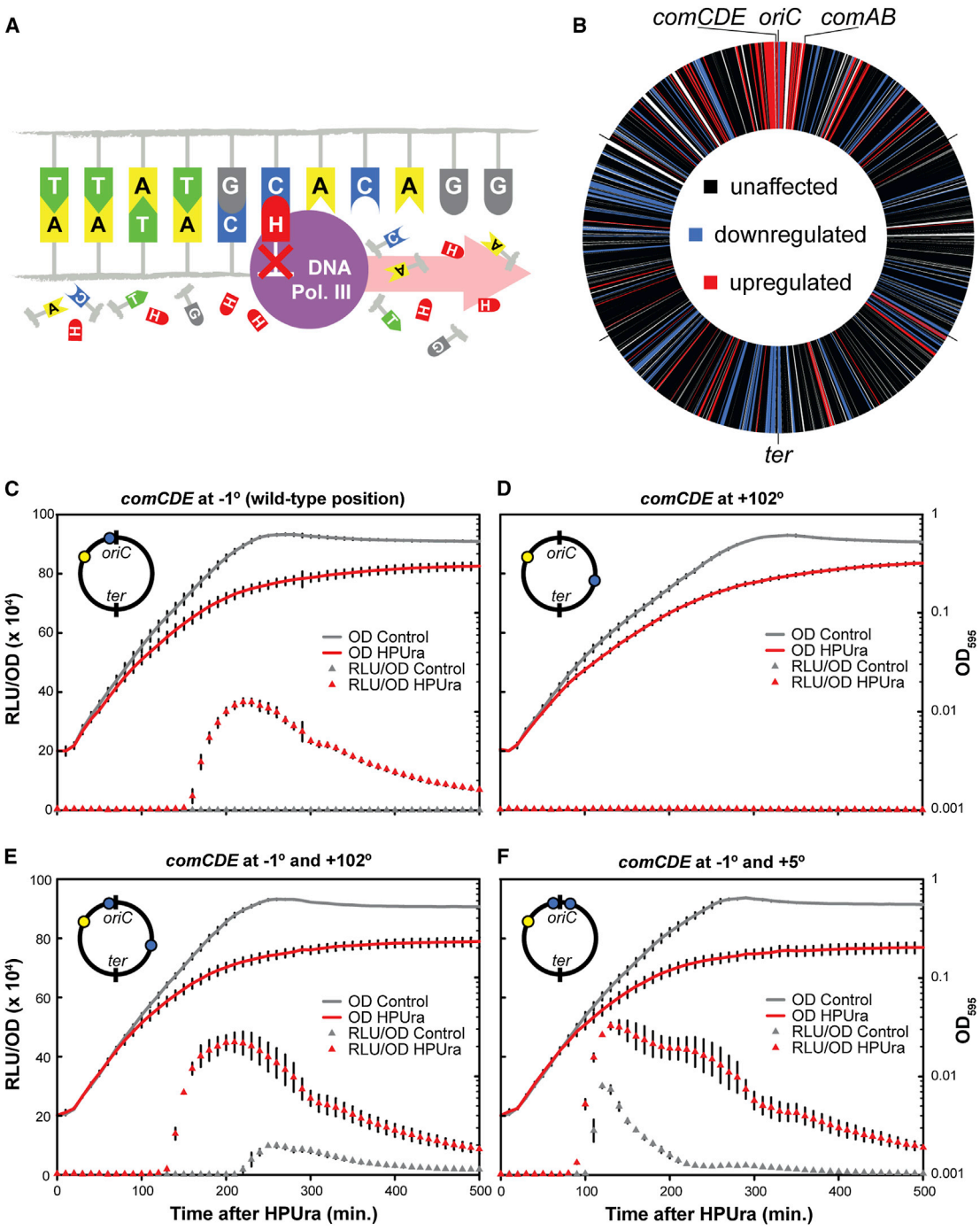

Figure 2. Replication Stress Induces Competence

(A) HPUra is intracellularly converted to a deoxyguanosine triphosphate (dGTP) analog (red shape) that cannot be incorporated in DNA; it competes with dGTP (gray shape) for reversible binding to DNA polymerase type III, thereby stalling the replication fork (Brown, 1970).

(B) The transcriptional response to HPUra is plotted on a circular representation of the chromosome. Significantly upregulated genes are colored red, unaffected genes in black, and downregulated genes in blue (cutoff p value = 0.01; see also Table S2).

(C–F) Strains MK134 (*ssbB_luc*, wild-type *comCDE*) (C), MK145 (*ssbB_luc*, Δ*comCDE,* Δ*bgaA*::*comCDE*) (D), MK139 (*ssbB_luc*, wild-type *comCDE,* Δ*bgaA*::*comCDE*) (E), and MK184 (*ssbB_luc*, wild-type *comCDE, prsA-comCDE*) (F) were grown in medium with (red lines/symbols) or without (gray lines/symbols) 0.15 μg/ml HPUra; optical density (OD_{595}; right axis) and luciferase activity as relative luminescence units per OD (RLU/OD; left axis) were determined every 10 min. Averages of at least three replicates with the SEM are plotted. Insets show the approximate positions of *comCDE* (blue dot) and *ssbB_luc* (yellow dot) on the circular chromosome.

See also Figures S1 and S4 and Table S1.

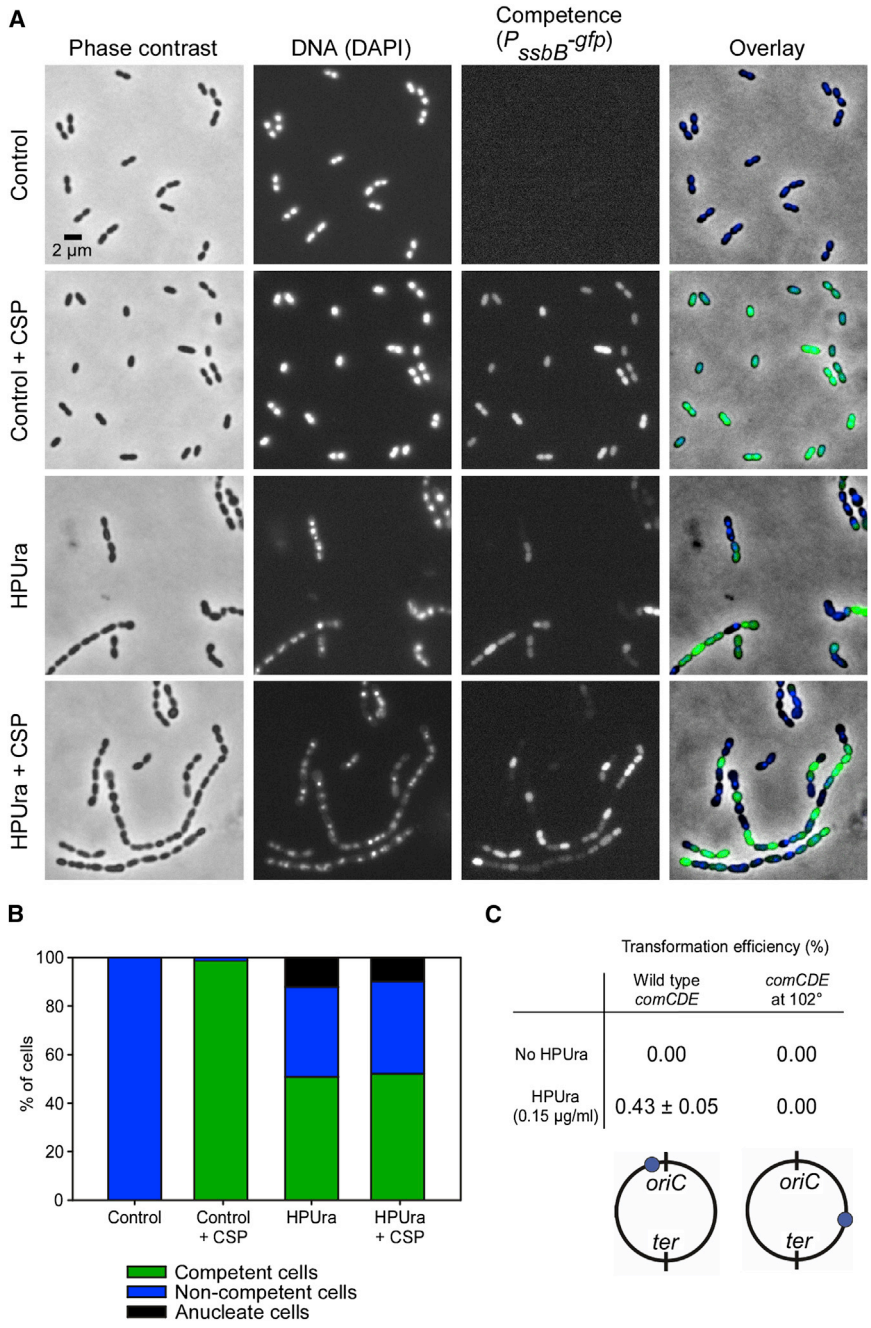

Figure 3. Single-Cell Analysis Shows that HPUra Induces Competence in All Viable Cells

(A) Representative micrographs are shown. In the overlays, GFP signal is in green and DAPI signal in blue. Scale bar = 2 μm. Competence is not activated in control cells grown in C+Y medium (pH 7.4), but when CSP is added, the competence pathway is activated in all cells as exemplified by high expression of GFP. When cells are grown in the presence of 0.15 μg/ml HPUra, competence is strongly activated in approximately 50% of the cells. A threshold GFP fluorescence value of 25% above background was used for counting of competent cells; thus, the actual percentage of competent cells will be underestimated.

(B) Fraction of competent cells and noncompetent cells (below the detection threshold) under different conditions. More than 500 cells were analyzed for each sample.

(C) Transformation efficiencies of untreated versus HPUra treated MK134 (wild-type *comCDE*) and MK145 (*comCDE* at 102°). Cells were incubated with 1 μg/ml of DNA containing the *rpoB*^{D489V} allele conferring resistance to rifampicin, and cells were plated either with (number of transformants) or without (total viable count) 4 μg/ml rifampicin to determine transformation efficiency. ± represents the error in transformation efficiency, obtained by propagation of uncertainties in colony counts.

chromosomal DNA, PCR product, plasmid DNA), and the integration site (Lee et al., 1998). Also, transformation is far from the only manifestation of competence in *S. pneumoniae*, because the competence regulon consists of over 100 genes, most of which are not required for transformation (Dagkessamanskaia et al., 2004; Peterson et al., 2004).

Antibiotic-Induced Replication Stress Upregulates Origin-Proximal Gene Expression

To find out how competence is triggered by HPUra-mediated replication stress, we examined the global transcriptional response under conditions that do not permit competence even in the presence of HPUra (low pH of the growth medium; Prudhomme et al., 2006). Cells were harvested 15 min after HPUra was added and total RNA was used for transcriptome analysis using DNA microarrays (see Experimental Procedures). Functional analysis of the transcription data did not show any significantly affected functional classes or known pathways. However, when the data were analyzed as a function of the genes' position on the chromosome, a significant portion of *oriC*-proximal genes were found to be upregulated (Figure 2B). Because such an effect may prevent the detection of affected functional pathways, the analyses were repeated leaving out the 10% of genes closest to *oriC*. Still, however, no significantly affected pathways were observed (Table S2).

To obtain a more detailed map of the transcriptome changes that occur when HPUra induces competence in *S. pneumoniae*, we grew cells in microtiter plates and monitored the competence response via the P_{ssbB}-*luc* reporter. After competence was initiated, total RNA was isolated for RNA sequencing (RNA-seq). Analysis of the data showed that most of the previously identified competence genes (Dagkessamanskaia et al., 2004; Peterson et al., 2004), including *comCDE*, *comAB*, *comX*,

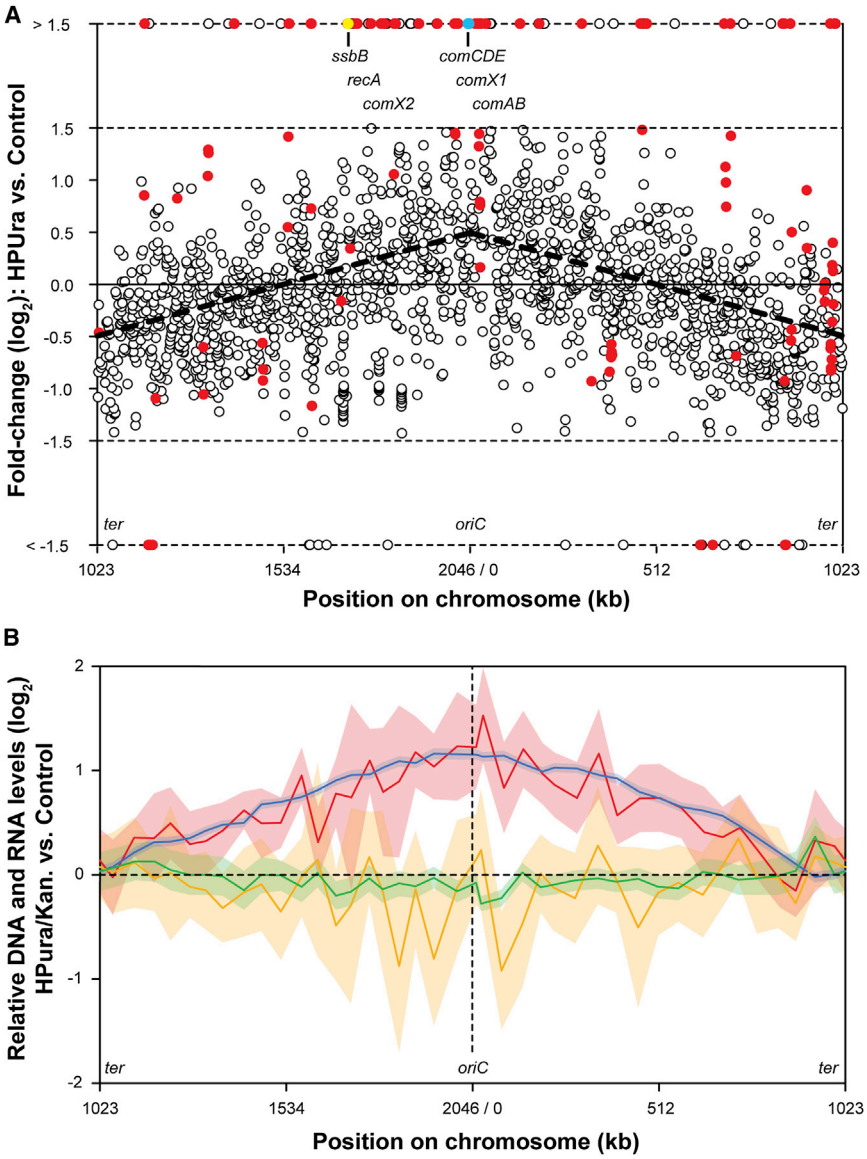

Figure 4. Gene Dosage Correlates with Gene Expression

(A) Fold change of gene expression (log_2) of *S. pneumoniae* treated with HPUra as determined by RNA-seq. Each circle represents the average fold change (y axis) of two biological replicates plotted as a function of the gene's position on the chromosome (x axis) with the origin region in the middle. Red filled circles represent previously identified competence genes (Dagkessamanskaia et al., 2004; Peterson et al., 2004). Genes with a log_2 fold change > 1.5 or < −1.5 are depicted on the top and bottom, respectively. The thick dashed line depicts a logarithmic trend line through the data, which was calculated while omitting the values for the known competence genes.

(B) Genome-wide marker frequency analysis using NGS. The median fold change on a sliding window of 51 genes is plotted for both the DNA (blue and green lines) and the RNA (red and orange lines) as a function of the central gene's position. The median absolute deviation from the median of these changes within the window is illustrated by the shaded regions. The gene copy number change (DNA) of HPUra-treated versus control is depicted by a blue line with the corresponding transcriptome (RNA) changes as a red line. The gene copy number change of kanamycin-treated versus control is depicted by a green line with the corresponding transcriptome changes as an orange line.

See also Figure S2.

recA, and *ssbB*, were highly upregulated by the addition of HPUra (Figure 4A). In line with the microarray data analysis, transcription of genes located close to the origin of replication was significantly upregulated in the presence of HPUra (Figure 4A). Together, this led us to postulate that upregulation of *oriC*-proximal genes is key to explaining competence induction upon replication stress, because the *comCDE* locus is located close to *oriC* (Figures 2B and 4A).

Upregulation of *oriC*-proximal genes can be traced back to the mode of action of HPUra, which slows down the elongation step of DNA replication (Figure 2A). If this type of stress does not block the initiation of new rounds of replication, it will lead to an increase in copy number of genes proximal to *oriC*. Alternatively, a transient pause of the replication fork without initiation of new rounds of DNA replication might also be sufficient to induce competence. To test between these two scenarios, we performed genome-wide marker frequency analysis of untreated versus HPUra-treated cells by next-generation sequencing (NGS). As shown in Figure 4B (blue line), a significant increase of origin-proximal gene copy numbers in the HPUra-treated cells was observed, demonstrating a shift in gene-dosage distribution. Importantly, the observed fold changes in DNA strongly correlate with the observed changes in the transcriptome (Figure 4B; compare red line with blue line). As a control for our assay, we also examined cells treated with kanamycin, an antibiotic that induces competence due to accumulation of misfolded proteins (Stevens et al., 2011). Indeed, we find a close correlation between gene dosage and transcript abundance in this case as well, and origin-proximal genes were not specifically upregulated by kanamycin (Figure 4B; green and orange lines for DNA and RNA, respectively).

To study whether transcription of *oriC*-proximal genes is in general upregulated in response to replication stress, we constructed two strains: one containing a transcriptionally isolated *luc* gene driven by a constitutive synthetic promoter (P_{syn}) at the *bgaA* locus (572 kb from *oriC*, 102°, strain DJS14) and the other carrying the same construct at 29 kb from *oriC* (5°, strain DJS15) (Figure S2A). Real-time luminometry assays showed

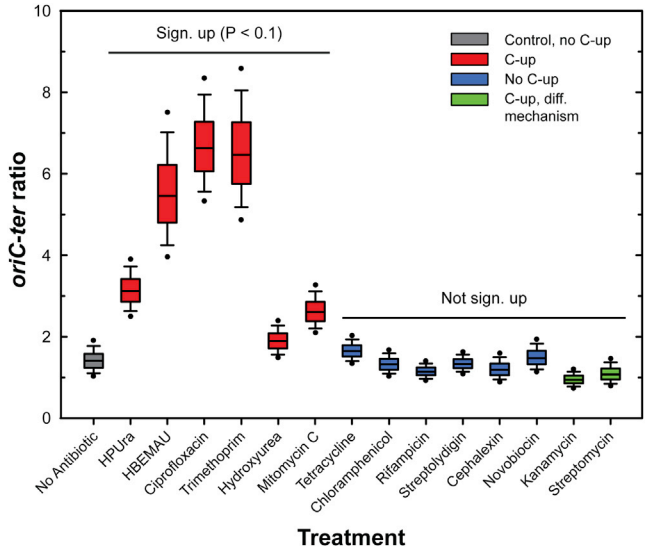

Figure 5. Antibiotic-Induced Shifts in Gene-Dosage Distribution Promote Competence

Boxplots represent *oriC-ter* ratios as determined by real-time qPCR. Dots represent the 5th and 95th percentile and whiskers represent the 10th and 90th percentile of data from Monte Carlo simulations. Strain DLA3 (P_{ssbB}-*luc*) was grown in medium without (control; gray box) or with the following antibiotics: HPUra (0.15 μg/ml), HBEMAU (0.3 μg/ml), ciprofloxacin (0.4 μg/ml), trimethoprim (0.7 μg/ml), hydroxyurea (608 μg/ml), mitomycin C (0.02 μg/ml), tetracycline (0.02 μg/ml), chloramphenicol (0.7 μg/ml), rifampicin (0.04 μg/ml), streptolydigin (300 μg/ml), cephalexin (1.25 μg/ml), novobiocin (1.25 μg/ml), kanamycin (28 μg/ml), and streptomycin (10 μg/ml). The color of the box indicates competence development (also see Figures S3 and S4). Note that the *oriC-ter* ratios as determined by real-time qPCR highly match the established *oriC-ter* ratios as determined by NGS for HPUra and kanamycin (see Figure 4).

that HPUra treatment had a larger effect on luciferase activity in the *oriC*-proximal than in the other construct (Figure S2B), confirming a general upregulation of *oriC*-proximal genes that is independent of their function.

Origin-Proximal Location of *comCDE* Is Required for Replication Stress-Induced Competence

If increased transcription of *oriC*-proximal genes (and thus *comCDE*) due to replication stress switches on the competence cascade, then moving the *comCDE* operon away from *oriC* should decrease this inducing effect. To test this, we placed the *comCDE* operon at the *bgaA* locus at 102° and removed it from its original locus. In line with our hypothesis, HPUra treatment could not induce competence in this strain (Figure 2D), demonstrating that the *oriC*-proximal location of *comCDE* is required to promote competence in response to replication stress at this pH. Furthermore, when a second copy of *comCDE* was integrated at 102° on the circular chromosome, the response to HPUra was augmented compared to the wild-type and cells even became competent without HPUra (Figure 2E). Finally, when a second copy of the *comCDE* operon was integrated close to *oriC*, at 5°, cells became competent without induction and HPUra activated the competence response even earlier (Figure 2F). Together, these experiments establish a causal link between *comCDE* copy number and induction of competence development.

All Antibiotics Causing Increased Origin Gene Copy Numbers Promote Competence

If a shift in gene-dosage distribution caused by HPUra can lead to competence induction, then this mechanism could be generalized to any antibiotic that causes an increase in *oriC*-proximal gene copy numbers, because only a slight increase in *comCDE* expression can already set in motion the ComE-autocatalytic loop and promote competence (Alloing et al., 1998; Figures 2E and 2F). To test this hypothesis, we performed marker frequency analysis using real-time quantitative PCR (qPCR) on cells treated with different antibiotics and checked whether they induced competence. For all antibiotics, we used a minimum inhibitory concentration required to inhibit the growth approximately 50% (MIC_{50}) (Figure S3). Strikingly, each antibiotic leading to an increased ratio of origin to terminus (*oriC-ter* ratio) also activated competence (Figures 5 and S3), and the origin-proximal location of *comCDE* highly contributed to this effect (Figure S4). This analysis now extends the known list of antibiotics that induce competence with HPUra, HBEMAU (N3-hydroxybutyl 6-[3′-ethyl-4′-methylanilino]-uracil [PolC inhibitor]; Tarantino et al., 1999), hydroxyurea (decreases the cellular pool of deoxyribonucleoside triphosphate via inhibition of ribonucleotide reductase), ciprofloxacin (DNA-gyrase inhibitor, see below), and trimethoprim (blocks dihydrofolate-reductase leading to reduced pools of thymidine). Kanamycin and streptomycin induced competence but did not lead to increased *oriC-ter* ratios. These antibiotics likely activate competence by increasing the concentration of misfolded proteins, thus occupying HtrA, as described previously (Stevens et al., 2011). Protein synthesis inhibitors tetracycline and chloramphenicol, RNA-polymerase inhibitors rifampicin and streptolydigin, and cell-wall synthesis inhibitor cephalexin did not induce competence or increase the *oriC-ter* ratio (Figures 5 and S3). Interestingly, both ciprofloxacin (targeting GyrA) and novobiocin (targeting GyrB) inhibit the DNA-gyrase complex consisting of two dimers of GyrA and GyrB (Lewis et al., 1996), but only addition of ciprofloxacin caused increased origin-gene copy numbers and subsequently induced competence (Figure 5). This example further exemplifies the requirement of increased origin-proximal-gene copy numbers for competence activation.

Origin-Proximal Location of *com* Genes Is Conserved

To establish whether the origin-proximal location of *com* genes is conserved, we examined the genomes of streptococci that have the same set of regulatory genes (*comCDE* and *comAB*) as the here-studied *S. pneumoniae* strain. Without exception, the *com* genes closely colocalized with *oriC* (Table S1). Notably, *S. thermophilus,* in which competence is regulated by *comRS* instead of *comCDE*, downregulates competence when confronted with MMC (Boutry et al., 2013). In line with our model, the *comRS* operon is located relatively far from *oriC* at 272 kb (Table S1). Thus, antibiotics that target DNA replication lead to shifted gene-dosage distributions, and this mechanism may be responsible for triggering competence in

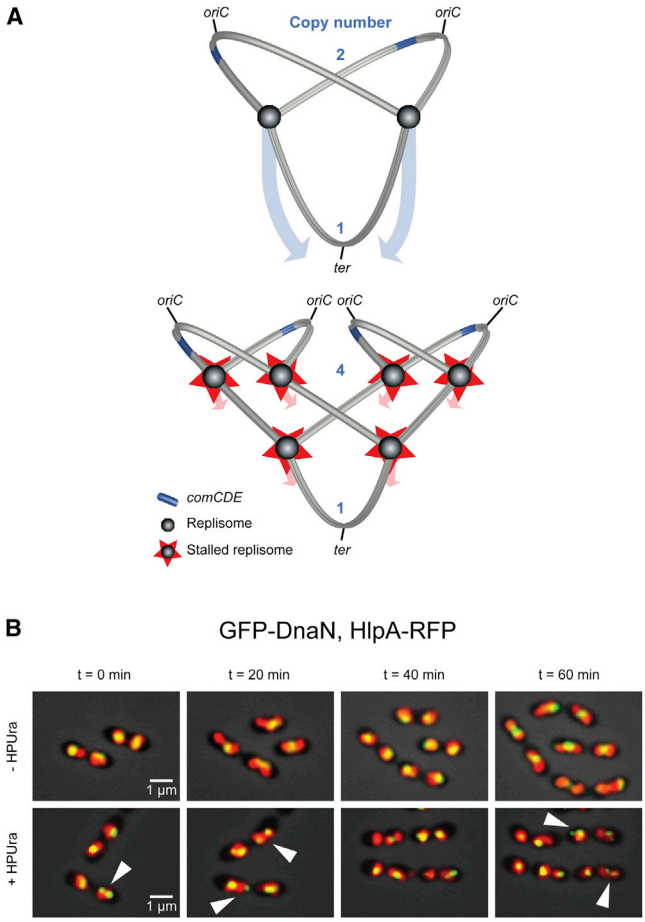

Figure 6. Stalled Replication Forks Increase *oriC*-Proximal Gene Dosage

(A) Model for induction of competence by antibiotics. Bacterial chromosomes are replicated bidirectionally from *oriC*. Replisomes are depicted by spheres. After initiation of DNA replication, the *oriC-ter* ratio is 2 (top image). In the presence of replication stress (bottom image), the replisomes frequently stall (red stars) and the elongation rate is reduced. Because new rounds of DNA replication are initiated, the *oriC-ter* gene-dosage ratio increases (to 4 in this example). More copies of *oriC*-proximal genes such as *comCDE* (blue line) subsequently lead to increased transcript levels. In the case of *comCDE*, this increased number of gene copy is sufficient to switch on the autocatalytic loop leading to competence development.

(B) Time-lapse microscopy of double-labeled strain DJS35 (HlpA-RFP, GFP-DnaN) grown without (top) or with (bottom) 0.05 μg/ml HPUra. Overlays between phase-contrast (gray), HlpA-RFP signal (red), and GFP-DnaN signal (green) are shown. Time point t = 0 corresponds to approximately 30 min after cells were exposed to HPUra. Arrows point to nucleoids with multiple replisomes. Scale bar represents 1 μm.

all *comCDE*-containing streptococci (Figure 6A). Gram-negative *Helicobacter pylori* and *Legionella pneumoniae* also become competent upon antibiotic-induced replication stress, but the genes and mechanisms involved in their competence regulation are unknown (Charpentier et al., 2011; Dorer et al., 2010). The here-described gene-dosage effect might play a role in this process if the master regulators of competence in these species are located near *oriC*.

Our model implies that the observed increase in relative amounts of origin-proximal genes upon exposure of cells to antibiotics targeting DNA replication requires the presence of multiple active replication machineries (replisomes). To test this, we performed time-lapse fluorescence microscopy of a *S. pneumoniae* strain harboring a red fluorescent protein (RFP) fused to the histone-like protein HlpA and a GFP fused to DnaN, the β clamp of the replication machinery. The HlpA-RFP fusion is a good proxy for visualizing the nucleoid in live cells (Kjos and Veening, 2014), while the GFP-DnaN fusion will only form distinct foci in actively replicating cells (Su'etsugu and Errington, 2011). As shown in Figure 6B, cells grown in the presence of subinhibitory amounts of HPUra contain more active replisomes per nucleoid compared to untreated cells (9% of nucleoids with >1 GFP-DnaN foci in normally growing cells versus 27% of nucleoids with >1 GFP-DnaN foci with HPUra; n > 80 nucleoids).

Antibiotics Targeting DNA Replication Increase Origin-Proximal Gene Copy Numbers in *B. cereus*, *S. aureus*, and *E. coli*

We wondered whether antibiotic-induced replication stress also leads to gene-dosage shifts in other bacteria. Therefore, we examined three pathogenic bacteria from different families (*B. cereus*, *S. aureus*, and *E. coli*). As shown in Figure 7A, the selected antibiotics targeting DNA replication also increased *oriC-ter* ratios in these bacteria. It was shown previously that hydroxyurea also causes increased *oriC-ter* ratios in *E. coli* (Odsbu et al., 2009). Together, these results demonstrate that a common molecular consequence of HPUra, hydroxyurea, trimethoprim, ciprofloxacin, and MMC is replication fork stalling.

Ciprofloxacin, MMC, and Trimethoprim Induce Origin-Specific Transcriptional Responses in Bacteria

We hypothesized that the observed increased gene copy number of *oriC*-proximal genes in response to replication stress might be responsible for many previously documented global antibiotic-induced transcriptional responses in bacteria (e.g., Cirz et al., 2007; Gmuender et al., 2001; Goranov et al., 2005, 2006; Khil and Camerini-Otero, 2002; Marrer et al., 2006; Yamane et al., 2012; for a review, see Wecke and Mascher, 2011). In *S. aureus*, for instance, it was documented that transcription of more than 500 genes were significantly changed in response to ciprofloxacin (Cirz et al., 2007). However, differential expression of only 16 genes could be attributed to the SOS response. Likewise, a study on the effects of MMC in *Listeria monocytogenes* showed differential expression of more than 70 genes even in the absence of the SOS response (in a *recA* mutant) (van der Veen et al., 2010). The molecular mechanism underlying these global changes in the transcriptome of *S. aureus* and *L. monocytogenes* in response to these antibiotics remained elusive. Upon re-analysis of the microarray data published by Cirz et al. (2007) and van der Veen et al. (2010), upregulated transcription of *oriC*-proximal genes was observed in both *S. aureus* and *L. monocytogenes* when treated with these DNA replication targeting antibiotics (Figures 7B and 7C). A similar trend was observed in *Campylobacter jejuni* and *S. thermophilus* treated with these antibiotics

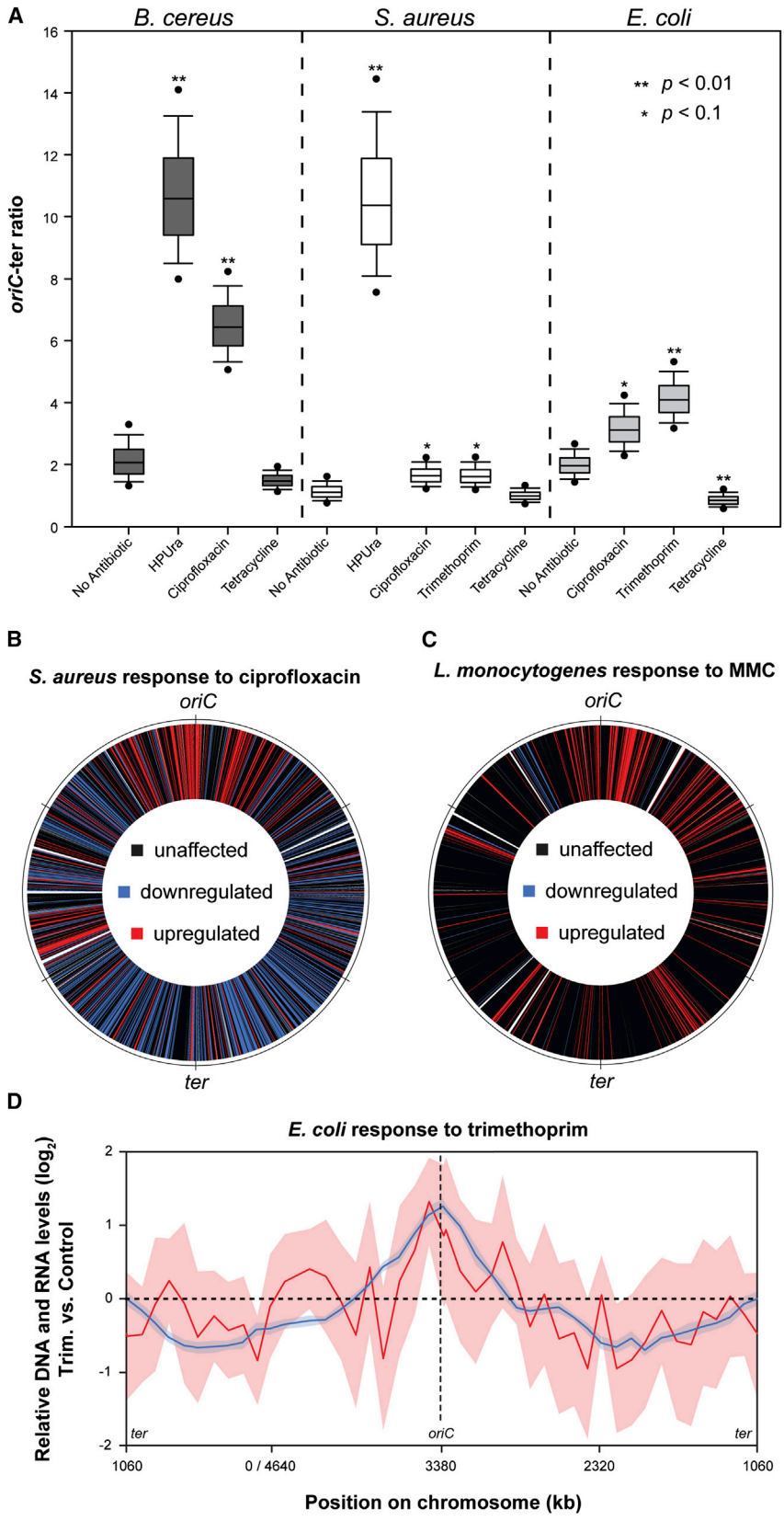

Figure 7. Antibiotics Affecting DNA Replication Lead to Increased Origin-Proximal Gene Copy Numbers and Subsequent Transcriptome Changes in Bacteria

(A) *B. cereus*, *S. aureus*, and *E. coli* were grown in medium with or without the indicated antibiotic (see also Figure S5). Boxplots represent *oriC-ter* ratios as determined by real-time qPCR, dots represent the 5th and 95th percentile, and whiskers represent the 10th and 90th percentile of data from Monte Carlo simulations. Note that *B. cereus* is resistant to trimethoprim and *E. coli* to HPUra.

(B and C) Transcription of origin-proximal genes is upregulated in response to (B) ciprofloxacin in *S. aureus* and to (C) MMC in *L. monocytogenes*. Data from Cirz et al. (2007) and van der Veen et al. (2010) are plotted on a circular representation of the corresponding chromosome. Significantly upregulated genes (cutoff p value = 0.01; see also Table S3) are colored red, unaffected genes are black, and downregulated genes are blue.

(D) A sublethal concentration of trimethoprim induces a gene-dosage shift in *E. coli*. Cells were treated with 0.5 μg/ml of trimethoprim and the chromosomal DNA and total RNA were isolated and sequenced as described in Experimental Procedures. The median fold change on a sliding window of 101 genes is plotted for both the DNA (blue line) and the RNA (red line) as a function of the central gene's position, with the origin region in the middle. Note that the *E. coli* origin of replication is located at approximately 3,380 kb of the annotated genome. The median absolute deviation from the median of these changes within the window is illustrated by the shaded regions.

(Table S3). Surprisingly, when we re-analyzed a data set of *E. coli* treated with 25 μg/ml of trimethoprim, we could not find any significant upregulation of origin-proximal genes (Sangurdekar et al., 2011). Because we used only 0.5 μg/ml of trimethoprim for our marker frequency analysis (Figure 7A), we wondered if the difference in antibiotic concentration could be responsible for the discrepancy. Therefore, we performed genome-wide marker frequency and transcriptome analysis using NGS on *E. coli* cells treated with 0.5 μg/ml of trimethoprim. In line with our real-time qPCR data, the relative gene dosage of origin-proximal genes was increased by the exposure of sublethal concentrations of trimethoprim and the transcriptome, analyzed by RNA-seq, changed accordingly (Figure 7D). Besides the increased expression of origin-proximal genes, several other stress responses were activated as reported previously by Sangurdekar et al., including the nucleotide biosynthetic pathway and the SOS response (data not shown). Together, the here-uncovered molecular mechanism of gene-dosage shift upon antibiotic treatment can largely explain the global changes in transcription.

DISCUSSION

Here, we demonstrate that most antibiotics targeting DNA replication in bacteria, either directly (like HPUra) or indirectly (like ciprofloxacin and trimethoprim) cause stalled replication forks while DNA replication initiation continues. This results in an increase in copy numbers of genes close to the origin of replication and subsequent global changes in transcription (Figures 2, 4, 5, 6, and 7). The here-described mechanism is general for bacteria, because it is a direct consequence of replication fork stalling. In the case of *S. pneumoniae*, we show that this shifted gene-dosage results in activation of the competence pathway (Figures 2, 3, 4, and 5), which in turn allows the uptake of foreign DNA and thereby increases the chance of acquiring antibiotic resistance. Some of the antibiotics we tested are commonly used to treat pneumococcal infections. These insights might therefore guide medics in their choice of antibiotics, particularly for patients with a history of antibiotic resistance.

Importantly, we show that antibiotics that target DNA replication lead to shifted gene dosages in not only *S. pneumoniae* (Figures 2 and 4) but also *E. coli*, *B. cereus*, and *S. aureus* (Figure 7). Re-analysis of several publicly available transcriptome data sets showed that MMC and ciprofloxacin also cause significant upregulation of *oriC*-proximal genes in *S. aureus*, *L. monocytogenes*, *S. thermophilus*, and *C. jejuni* (Figure 7; Table S3). Interestingly, while MMC also causes a relative increase in origin-proximal gene expression and gene dosage in *Bacillus subtilis*, this effect was not observed when *B. subtilis* cells were treated with 38 μg/ml of HPUra (Goranov et al., 2006). Because we used 0.15 μg/ml of HPUra to slow down DNA replication in *S. pneumoniae*, this might indicate that the concentration and duration of the antibiotic treatment is also important in establishing the shift in gene dosage. Indeed, *E. coli* treated with bactericidal concentrations of trimethoprim did not present an upregulation of origin-proximal gene expression (Sangurdekar et al., 2011), while addition of bacteriostatic concentrations did generate this response (Figure 7D). Thus, when replication is blocked completely by the addition of high amounts of antibiotics, replication elongation cannot proceed and the gene-dosage shift and subsequent origin-proximal transcriptome changes do not occur. For *E. coli*, it has been shown that high bactericidal concentrations of certain antibiotics can induce cell death, mediated by reactive oxygen species (ROS) (e.g., Davies et al., 2009; Kohanski et al., 2007), although this view has recently been challenged (Keren et al., 2013; Liu and Imlay, 2013). Our study highlights that the experimental conditions and antibiotic concentrations used can add to the complexity of the bacterial stress response, and it will be interesting to see how much the here-described gene-dosage effect plays a role in the previously reported antibiotic-induced ROS-dependent cell death of many bacteria.

Genes involved in transcription and translation often colocalize with the origin of replication in bacteria, likely because this allows rapid growth, as the bacterium can take maximum advantage of the transient difference in gene dosage after initiation of bidirectional replication (Couturier and Rocha, 2006). Also, for the developmental process of sporulation in *B. subtilis,* the *oriC*-proximal location of certain sporulation genes is important to allow for well-controlled and compartmentalized gene expression (Wu and Errington, 1994; Eldar et al., 2009). Here, we find that the origin-proximal location of the *com* regulatory genes provides a built-in mechanism by which cells can activate competence to robustly respond to antibiotic-induced replication stress. Future studies will reveal whether the *oriC*-proximal location is conserved also for other genes involved in response to replication stress (e.g., SOS-like responses) in bacteria.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Growth Conditions, Transformation, and Luminescence Assays

Bacterial strains and plasmids used in this study are listed in Table S4, and oligonucleotides are listed in Table S5. *S. pneumoniae* was generally grown in complex C+Y medium (Martin et al., 1995) at 37°C. Luminescence assays were performed with a Tecan Infinite 200 PRO luminometer at 37°C. Every 10 min, optical density 595 (OD_{595}) and luminescence (expressed in relative luminescence units [RLU]) was measured. Expression of the *luc* gene results in the production of luciferase and thereby in the emission of light when the medium contains luciferin (Prudhomme and Claverys, 2005). To calculate the transformation efficiency, 1 μg/ml of PCR product of a 1.7 kb DNA fragment containing the $rpoB^{D489V}$ allele (encoding the RNAP β-subunit) that confers resistance to rifampicin (Martín-Galiano and de la Campa, 2003) was added in the wells prior to competence initiation as measured by the *ssbB-luc* reporter. Serial dilutions were plated either with or without 4 μg/ml rifampicin (>300 MIC), and the transformation efficiency was calculated by dividing the number of transformants by the total viable count. Detailed growth conditions for the different assays are described below and in Extended Experimental Procedures.

DNA Microarrays

Two samples of DLA3 (*bgaA::*P_{ssbB}*-luc*) were grown as standing culture without aeration at 37°C to OD_{600} = 0.15 in 50 ml C+Y medium, adjusted to pH 7.0 with 1 M HCl. To one sample, HPUra (in 10 mM NaOH) was added (0.15 μg/ml final concentration). To the other sample, the same volume of 10 mM NaOH was added as a control. RNA was isolated and cDNA was made as described in Extended Experimental Procedures. Procedures described previously (van Hijum et al., 2005) were followed regarding microarrays and data analysis, where DyLight550 and DyLight650 take the role of Cy3 and Cy5, respectively. Additionally, after normalization, the tool OpWise

(Price et al., 2006) was used to analyze microarray data, taking into account operon predictions (Price et al., 2005) for higher sensitivity. For visualization of the upregulation of *oriC*-proximal genes, using the Microbial Genome Viewer (Kerkhoven et al., 2004) (Figure 2B), a cutoff p value of 0.01 was used to select affected genes. Publicly available transcriptome data were obtained from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/).

DNA and RNA Sequencing

For sequencing, samples of *S. pneumoniae,* strain DLA3 (*bgaA::*P_{ssbB}*-luc*) was grown to OD_{600} = 0.4 in 5 ml tubes and diluted 1:100 in fresh C+Y medium (pH 7.4). To study the effects of antibiotics, cells grown without antibiotics were compared to cells grown with 0.15 μg/ml HPUra or 28 μg/ml kanamycin. Cells were grown in microtiter plates and growth and competence development (*luc*-expression) was followed. For *E. coli*, overnight cultures of strain JM83 (a K-12 derivative) were diluted 1:100 in LB medium and grown in microtiter plates. For all the samples, when one-third of the maximum OD_{600} was reached, cells were harvested by centrifugation (7,500 rcf for 5 min) and frozen. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). For RNA isolation, cells were lysed by bead beating and RNA was purified using phenol-chloroform extractions and ethanol precipitations. DNA was removed from the sample with RNase-free DNase I (Fermentas) treatment for 45 min. Ribolock (Fermentas) was added to avoid RNA degradation.

For both DNA and RNA sequencing, all conditions were sampled in duplicate, except for kanamycin-treated *S. pneumoniae*, for which data were obtained from a single sample. Duplicate data showed very high correlation ($R^2 > 0.9$). Library preparation and whole-genome sequencing were performed by vertis Biotechnologie AG. For RNA-seq, ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Epicenter) prior to preparation of cDNA libraries. Sequencing of both DNA and cDNA libraries was performed with an Illumina HiSeq 2000 machine with 100 bp read length.

Sequence reads were mapped to either the *S. pneumoniae* D39 genome (NC_008533) or the *E. coli* K12, substr. MG1655 genome (NZ_AKBV01000001) using Rockhopper (McClure et al., 2013) for *S. pneumoniae* or Bowtie (Langmead and Salzberg, 2012) for *E. coli*. Data analysis is explained in more detail in Extended Experimental Procedures.

Re-Analysis of Transcriptional Response of Other Organisms to Ciprofloxacin and MMC

We re-analyzed microarray data sets describing the transcriptional response of other organisms than *S. pneumoniae* to antibiotics affecting DNA replication (Table S3). This was done to show that the upregulation of *oriC*-proximal genes by antibiotics affecting DNA replication can also explain previously unnoticed, misunderstood, or unexplained expression patterns in existing microarray data sets. The 10% of genes with the highest fold changes (in either direction) were considered. Of these changers, we looked how many of the upregulated ones were among the 100 *oriC*-proximal genes. To locate the origin of replication of these organisms, we looked for the region with the highest density of perfect DnaA-boxes (TT(A/T)TNCACA). A hypergeometric test was then used to calculate whether or not a significant number of *oriC*-proximal genes were upregulated in these experiments. Some of these data sets were visualized with the Microbial Genome Viewer (Kerkhoven et al., 2004) and plotted (Figures 7B and 7C).

oriC-ter Ratio Determination by Real-Time qPCR

Cells were grown as described above and in detail in Extended Experimental Procedures. In the real-time qPCR experiments, each 20 μl sample consisted of 8.8 ng of DNA, 0.6 pmol of each primer (Table S5), and 10 μl of 2x SYBR Green Supermix (Bio-Rad). Amplification was performed on a iQ5 Real-Time PCR Detection System (Bio-Rad). To find the amplification efficiencies, Monte Carlo simulations were performed with Mathematica (Wolfram Research); average C_T-values and their corresponding standard deviations were used to simulate 10,000 new sets of C_T-values, and with those the amplification efficiencies were computed for each set. From that population of possible efficiencies, averages and SDs were derived. Analysis of the real-time qPCR experiments for *oriC-ter* ratio determination was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), with the important difference that the earlier found amplification efficiencies were used to determine the fold-change per cycle, instead of assuming it to equal 2. As a reference, cells with an assumed *oriC-ter* ratio of 1 were used (see Supplemental Information). Uncertainties in *oriC-ter* ratios were also determined by Monte Carlo simulations.

Time-Lapse Fluorescence Microscopy

Time-lapse fluorescence microscopy was performed basically as described previously (de Jong et al., 2011; Kjos and Veening, 2014). More details are available in Supplemental Information.

ACCESSION NUMBERS

The GEO (http://www.ncbi.nlm.nih.gov/geo/) accession number for the microarray data reported in this paper is GSE46002. The GEO accession number for the NGS data reported in this paper is GSE54199.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.01.068.

AUTHOR CONTRIBUTIONS

J.S., M.K., L.A., and J.W.V. designed research and analyzed data; J.S., M.K., and L.A. performed research; and J.S. and J.W.V. wrote the paper.

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