

Cyclic di-GMP as an Antitoxin Regulates Bacterial Genome Stability and Antibiotic Persistence in Biofilms

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Abstract

Biofilms are complex bacterial communities characterized by a high persister prevalence, which contributes to chronic and relapsing infections. Historically, persister formation in biofilms has been linked to constraints imposed by their dense structures. However, we observed an elevated persister frequency accompanying the stage of cell adhesion, marking the onset of biofilm development. Subsequent mechanistic studies uncovered a comparable type of toxin-antitoxin (TA) module (TA-like system) triggered by cell adhesion, which is responsible for this elevation. In this module, the toxin HipH acts as a genotoxic deoxyribonuclease, inducing DNA double strand breaks and genome instability. While the second messenger c-di-GMP functions as the antitoxin, exerting control over HipH expression and activity. The dynamic interplay between c-di-GMP and HipH levels emerges as a crucial determinant governing genome stability and persister generation within biofilms. These findings unveil a unique TA system, where small molecules act as the antitoxin, outlining a biofilm-specific molecular mechanism influencing genome stability and antibiotic persistence, with potential implications for treating biofilm infections.

eLife assessment

This work describes how the toxin-antitoxin (TA) system, which uses the cyclic di-GMP as an antitoxin, controls both the persistence of antibiotics linked to biofilms and the integrity of the bacterial genome. The authors present **solid** evidence linking cyclic di-GMP and the toxin HipH. The work is **valuable** because it establishes the relationship between bacterial persistence and biofilm resilience, which lays a strong basis for future research on the formation of bacterial biofilms and antibiotic resistance.

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Introduction

The prevalence of chronic and recurrent microbial infections has been significantly attributed to the formation of biofilms - complex, surface-attached bacterial communities (Costerton et al, 1999 [↗](#); Hall-Stoodley et al, 2004 [↗](#); Lewis, 2005 [↗](#)). In biofilms, bacteria exhibit reduced antibiotic sensitivities, a phenomenon occurring independently of the presence of resistance determinants on genetic elements (Ceri et al, 1999 [↗](#)). This antibiotic insensitivity is linked to the elevated prevalence of persister cells in biofilms, with rates ranging from 10 to 1000 times higher compared to their planktonic counterparts (Lewis, 2005 [↗](#); Yan & Bassler, 2019 [↗](#)). Persister cells, recognized as phenotypic variants in an isogenic population, demonstrate a remarkable ability to withstand antibiotic treatment and, intriguingly, can resuscitate once the antibiotic pressure is alleviated (Balaban et al, 2004 [↗](#); Bigger, 1944 [↗](#); Lewis, 2007 [↗](#); Zhou et al, 2023 [↗](#)). These characteristics collectively position persister cells as key contributors to the perpetuation of persistent infections of biofilms.

Despite the high frequency in biofilms and critical roles in both clinical context and evolution processes, the mechanisms underlying persister formation in biofilms are the subject of intense debate. The effects induced by high density, including nutrient and oxygen deficiency, and quorum sensing within dense structure of developed biofilms, have long been considered as primary factors leading to persister formation (Ganesan et al, 2016 [↗](#); Nguyen et al, 2011 [↗](#); Yan & Bassler, 2019 [↗](#); Yan et al, 2018 [↗](#)). This association originally stems from the hypothesis of limitations in nutrient, oxygen, or antibiotic penetration into the dense structures. Nevertheless, recent challenges to this hypothesis have arisen from studies indicating that the matrix mesh size of biofilms is significantly larger than the size of molecules such as nutrients, oxygen, and antibiotics (Ganesan et al, 2016 [↗](#); Yan & Bassler, 2019 [↗](#); Yan et al, 2018 [↗](#)). Notably, most antibiotics do not strongly interact with biofilm matrix components (Spoering & Lewis, 2001 [↗](#)). An alternative perspective emerges from observations that cells in mature biofilms exhibit metabolic activity similar to cells in the stationary phase (Nguyen et al, 2011 [↗](#); Walters et al, 2003 [↗](#); Waters et al, 2016 [↗](#)), during which the rate of persister cell formation significantly surpasses that of cells in the exponential phase. This phenomenon is likely attributed to nutrient exhaustion in the local environment surrounding mature biofilms (Nguyen et al, 2011 [↗](#); Walters et al, 2003 [↗](#); Waters et al, 2016 [↗](#)). Meanwhile, some findings propose the involvement of specific genes in persister cell formation in biofilms (Zhao et al, 2013 [↗](#)), many of these observations are derived from research conducted under planktonic (free-swimming) conditions with bulk cells (Yan & Bassler, 2019 [↗](#)).

Recent research has shed light on the critical role of small metabolic molecules in regulating the formation and resuscitation of persister cells, including persister cells in biofilms, providing valuable insights into the complex mechanisms of bacterial persistence. Guanosine pentaphosphate and tetraphosphate ((p)ppGpp), key alarmones in the bacterial stringent response, have been shown to mediate ribosome dimerization, leading to persistence, while their degradation triggers resuscitation. This dual function of (p)ppGpp underscores its significance in both initiating and terminating the persister state (Song & Wood, 2020 [↗](#); Wood & Song, 2020 [↗](#); Wood et al, 2019 [↗](#)). Additionally, the interaction between cyclic AMP (cAMP) and the cAMP receptor protein (CRP) has been identified as a crucial regulator of bacterial persistence. The cAMP-CRP complex acts as a global regulator, influencing persistence through modulation of cellular metabolism and regulation of stress responses (Mok et al, 2015 [↗](#); Niu et al, 2024 [↗](#)). These molecular mechanisms offer a more nuanced understanding of persister cell dynamics.

Studying a biofilm during its early developmental stage offers a unique vantage point, minimizing the impact of dense structure or nutrient exhaustion, thus allowing for a controlled exploration of intrinsic factors influencing persister cell formation. Cell-surface adhesion is the critical initiation step in the early stage of biofilm development, encompassing both reversible and irreversible

processes (Berne et al, 2018 [↗](#); Tolker-Nielsen, 2015 [↗](#)). During reversible adhesion, planktonic bacteria utilize surface appendages such as flagella and pili, fostering a weak attachment through van der Waals forces (Ren et al, 2018 [↗](#)). Subsequently, these reversible adhered cells sense environmental cues linked to the surface, activating signaling pathways that lead to the synthesis of 3',5'-cyclic diguanylic acid (c-di-GMP). Elevated c-di-GMP levels function as a secondary messenger, orchestrating a shift in bacterial behavior by influencing the expression of genes crucial to biofilm development. This signaling cascade propels the transition from reversible to irreversible adhesion, wherein bacteria establish microcolonies on the surface, solidifying a more stable and irreversible connection (Jenal et al, 2017 [↗](#); Valentini & Filloux, 2016 [↗](#)).

To explore the intrinsic factors influencing persister cell formation during the early stages of biofilm development, we employed a static biofilm system for our investigation (Fig. 1A [↗](#)). This system is widely employed in research to scrutinize the pivotal processes of initial adherence during biofilm formation (Biswas & Mettlach, 2019 [↗](#); Merritt et al, 2005 [↗](#); Seviour et al, 2021 [↗](#); Sung et al, 2022 [↗](#)). In our initial study, we noted an increased persister frequency coinciding with the step of cell adhesion. This observation prompted the hypothesis that elevated c-di-GMP levels in individual cells potentially facilitate the formation of persister cells within biofilms. Further investigation substantiated this hypothesis, demonstrating that the elevation in c-di-GMP indeed promotes the formation of persister cells. Our detailed analysis uncovered the role of c-di-GMP as an antitoxin, exerting control over both the expression and genotoxicity of HipH, thereby regulating genome stability and persister cell formation. These findings propose a biofilm-specific mechanism for antibiotic persistence. Notably, targeting this toxin-antitoxin system effectively inhibited drug tolerance in Uropathogenic *Escherichia coli* (UPEC) infections, presenting promising therapeutic strategies against chronic and relapsing infections.

Results

An increased propensity of persister formation upon cell adhesion

To enhance the clinical relevance of our study, we employed UPEC, the predominant cause of urinary tract infections (UTIs) and responsible for approximately 80% of cases. UPEC has a notable ability to form biofilms on various surfaces, including catheter materials, bladder epithelial cell walls, and within bladder cells (Supplementary Information). In our investigation, we selected the clinical isolate UPEC16. This strain demonstrates a significant phylogenetic similarity to established UPEC reference strains such as CFT073 and UTI89 (Fig. S1A [↗](#)). Moreover, it displays sensitivity to a wide range of antibiotics and possesses the capability for surface adhesion. To culture UPEC16 cells in the static biofilm system, exponential phase cells with an optical density (OD₆₀₀) of 0.3 were diluted in 10-fold in fresh media, followed by static incubation in microtiter dishes for varying time intervals. Planktonic bacteria were then gently washed away, leaving adhered cells for further investigation. In the persister counting assay, both the remaining adhered cells and the removed planktonic cells were exposed to bactericidal antibiotics (ampicillin, 150 µg/ml, 10-fold of MIC) for varying durations. Subsequently, persister counting was conducted through plating.

Biphasic killing curves revealed distinct patterns. When cells were subjected to the same duration of static culture, the persister rate within the adhered population notably surpassed that observed in the planktonic population (Fig. 1B-D [↗](#)). Additionally, the persister rate within adhered populations exhibited a positive correlation with the prolonged duration of static culture (Fig. 1B-D [↗](#)). In contrast, the persister rate within planktonic populations displayed little variation with an extended duration of static culture (Fig. 1B-D [↗](#)). These observations underscore the crucial role of cell adhesion in promoting persister cell formation during the early stages of biofilm

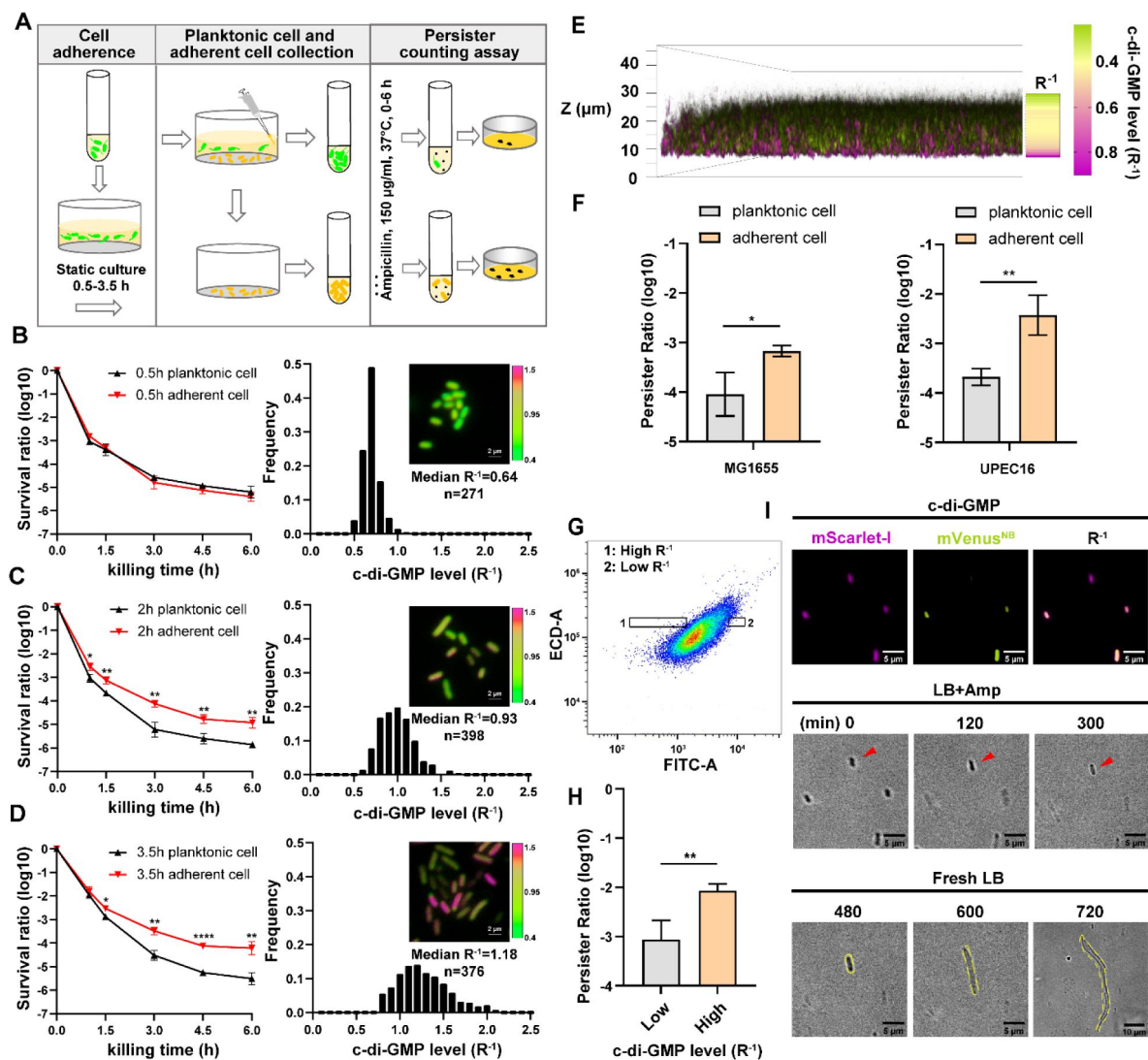


Figure 1.

Cell adhesion promotes elevation of c-di-GMP levels and persister formation.

(A) Schematic overview of the static biofilm system designed to investigate intrinsic factors influencing persister cell formation during the early stages of biofilm development. (B-D) Left panel: the biphasic killing curves of planktonic cell subpopulation and surface-adherent cell subpopulation from static biofilm system at the culture time of 0.5 h (B), 2h (C) or 3.5 h (D), respectively (n=3). Right panel: Frequency distribution of c-di-GMP levels (R^{-1} score) within the surface-adherent subpopulation from the same static biofilm system and time points. Insert fluorescent images provide representative illustrations of c-di-GMP levels in adherent cells, measured using the c-di-GMP sensor system (Fig. S1B) integrated into *E. coli* cells. Scale bar, 2 µm. R^{-1} score was determined using the fluorescent intensity of mVenus^{NB} and mScarlet-I in the system. (E) Spatial heterogeneity in c-di-GMP levels (R^{-1} score) within a 24-hour static biofilm in MG1655 strain captured through confocal microscopy. (F) The persister ratio in planktonic cell subpopulation and surface-adherent cell subpopulation collected from the 24-hour static biofilms (ampicillin, 150 µg/ml, 3-hour at 37°C with shaking at 220 rpm) (n=3). (G-H) The persister ratio (n=3) (H) in High- R^{-1} or Low- R^{-1} subpopulations sorted from the 24-hour static biofilms in MG1655 strain (ampicillin, 150 µg/ml, 3-hour at 37°C with shaking at 220 rpm) (G). (I) Representative time-lapse images of the persister assay using cells with different R^{-1} values. The MG1655 cells resuspended from 24-hour static biofilm were imaged on a gel pad, and subsequently treated with LB broth containing 150 µg/ml ampicillin, imaged over 6-hour at 35°C and then allowed to resuscitate in fresh LB. Scale bar, 5/10 µm, as indicated. Error bars represent standard deviations of biological replicates. Significance was ascertained by two-tailed Student's t test. Statistical significance is denoted as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

development. This raises questions about established ideas linking persister formation mainly with the dense structure of biofilms, suggesting a need to reconsider the factors that influence persister dynamics during biofilm initiation."

The correlation between heightened c-di-GMP levels and increased persister frequency

The increase in c-di-GMP levels is a distinctive feature of cell-surface adhesion, marking the transition of cells from a planktonic lifestyle to a sessile one (Fernandez et al, 2020 [↗](#); Jones et al, 2015 [↗](#); Valentini & Filloux, 2016 [↗](#)). Building on our observations of increased persister rates upon cell adhesion, we assumed that elevated c-di-GMP levels might play a role in promoting persister cell formation. To investigate this hypothesis and discern the relationship between c-di-GMP levels and persister rates, we introduced a ratiometric fluorescent c-di-GMP sensing system (Vrabioiu & Berg, 2022 [↗](#)) into the UPEC16 strain. MrkH is a transcription factor derived from *Klebsiella pneumoniae*. The system utilizes the conformational changes that occur in MrkH upon binding to c-di-GMP by strategically inserting it into mVenus^{NB} between residues Y145 and N146, resulting in the creation of the biosensor referred to as mVenus^{NB}-MrkH. This strategic integration results in a ratiometrically reduced fluorescence signal when the biosensor is bound to varying levels of c-di-GMP. Furthermore, a red fluorescent protein mScarlet-I is co-expressed under the regulation of the *proC* promoter (Davis et al, 2011 [↗](#)), enabling the normalization of the system's expression level. Consequently, the inverse of the ratio between mVenus^{NB}-MrkH and mScarlet-I (R^{-1}) exhibits a direct correlation with the intracellular level of c-di-GMP, establishing a reliable indicator for monitoring c-di-GMP dynamics in UPEC16 (Fig. S1B [↗](#)).

By monitoring the fluorescent intensity of the system at the single-cell level under a time-lapse microscope, we observed the dynamic activation of c-di-GMP during the process of cell adhesion. We imaged subpopulations on the surface at varied intervals. In the initial 30 minutes following static incubation in microtiter dishes, the adhered subpopulation displayed a sharply peaked R^{-1} distribution with a low median of 0.64 (Fig. 1B [↗](#)). As the attachment duration increased to 2 hours, the R^{-1} distribution broadened, accompanied by a higher median of 0.93 (Fig. 1C [↗](#)). Further extension to 3.5 hours resulted in a continued broadening of the R^{-1} distribution, accompanied by an even higher median of 1.18 (Fig. 1D [↗](#)). Meanwhile, the R^{-1} distribution of the planktonic subpopulation from the same culture system and time points remained low, with a median of 0.31 to 0.35, respectively (Fig. S1C [↗](#)-E [↗](#)). These findings replicate the observed positive correlation between c-di-GMP production and attachment duration. Moreover, the results suggest a positive association between c-di-GMP levels and the persister rate (Fig. 1B-D [↗](#)).

Microcolonies emerged on the surface of microtiter dishes when the duration of static culture extended to 24 hours (Fig. 1E [↗](#)). We employed confocal laser scanning microscopy to monitor spatial distribution of c-di-GMP signals within these microcolonies. This investigation revealed a stratified pattern of c-di-GMP levels across the cross-section of the static biofilms (R^{-1} , Fig. 1E [↗](#)), highlighting the intricate regulatory role of this signaling molecule in shaping bacterial behavior and biofilm structure. Notably, cells with the highest c-di-GMP levels were surface attached, predominantly situated at the bottom layers of the biofilm (Fig. 1E [↗](#)). To further investigate this observation, we isolated the surface attached cells and unattached cells in the biofilms for persister counting assay. The results demonstrate that this spatial distribution aligned with a subpopulation that exhibited the highest persister rate (Fig. 1F [↗](#)). Furthermore, we resuspended the cells within the static biofilms and used FACS to isolate subpopulations with varying levels of c-di-GMP (Fig. 1G [↗](#)). An antibiotic killing assay revealed that subpopulations with higher concentrations of c-di-GMP had a higher frequency of persister cells (Fig. 1H [↗](#)). Time-lapse imaging also revealed that cells with high intracellular c-di-GMP levels survived antibiotic treatment and resuscitated to form microcolonies (Fig. 1I [↗](#)). These findings collectively suggest that increased levels of cellular c-di-GMP play a crucial role in promoting persister formation.

Heightened c-di-GMP levels promotes persister formation

To investigate the involvement of c-di-GMP in promoting the transition of persister phenotype in biofilms, we first constructed a UPEC16 strain with the ability to degrade c-di-GMP in a controlled manner. This was achieved through the overexpression of PdeH, an efficient c-di-GMP phosphodiesterase in *E. coli*, under the control of the araBAD promoter (pBAD::pdeH) (Pesavento et al, 2008 [↗](#)). Inducing PdeH expression before cell attachment disrupted cell adhesion, validating the efficacy of our system. Initiating with uninduced cells, we allowed them to adhere to a surface in a microliter dish (**Fig. 2A** [↗](#)). After a 2-hour incubation, we observed robust cell adhesion similar to the vector control, reflected in comparable c-di-GMP R^{-1} medians. Following this initial observation, we replaced the old medium with fresh medium containing arabinose (0.2%) for PdeH induction. After an additional 30 minutes of incubation, we noted a substantial decrease in c-di-GMP R^{-1} levels in the PdeH overexpression strain (median of 0.75) compared to the vector control strain (median of 0.98) (**Fig. 2B-C** [↗](#)). Crucially, the biphasic killing curve showed significantly lower persister rates in adhered populations with PdeH overexpression compared to the vector control strain (**Fig. 2D** [↗](#)). These findings suggest that c-di-GMP plays a pivotal role in promoting persister cell formation during cell-surface adhesion.

In parallel, we engineered a UPEC16 strain overexpressing DgcZ, a highly efficient diguanylate cyclase responsible for synthesizing c-di-GMP, under the araBAD promoter (pBAD::dgcZ). In a consistent approach, we allowed the cells to adhere to a surface in a microliter dish before induction (**Fig. 2A** [↗](#)). After a 2-hour incubation, we observed cell adhesion comparable to the vector control, reflected in similar c-di-GMP R^{-1} medians. Subsequently, we replaced the old medium with fresh medium containing arabinose (0.2%) for DgcZ induction. Following an additional 30 minutes of incubation, we noted a substantial increase in c-di-GMP R^{-1} levels in the DgcZ overexpression strain (median of 1.72) compared to the vector control strain (**Fig. 2E** [↗](#)). Consistently, the biphasic killing curve indicated significantly higher persister rates in adhered populations with DgcZ overexpression compared to the vector control strain (**Fig. 2D** [↗](#)). These findings reinforce the critical role of c-di-GMP in promoting persister cell formation during cell-surface adhesion.

Toxin protein HipH acts downstream of c-di-GMP signaling in regulating persister formation in biofilms

Consistent with the observations in adherent cells, planktonic cells with DgcZ overexpression demonstrated a significant increase in the persister ratio (**Fig. 3A** [↗](#)). Yet, cells overexpressing DgcZ-mutant (DgcZ-m, G206A, G207A, E208Q), which lacks diguanylate cyclase activity (Boehm et al, 2009 [↗](#); Jonas et al, 2008 [↗](#)), did not manifest a parallel increase (**Fig. 3A** [↗](#)). These results reinforce that elevated levels of c-di-GMP play a key role in promoting persister cell transition. To explore the mechanisms by which elevated c-di-GMP levels promote bacterial persister formation, we employed a systematic screening approach using the *E. coli* Keio Knockout Collection (KEIO collection) (Baba et al, 2006 [↗](#)). Given that the K-12 strain, the background strain of the KEIO collection, might not be ideal for cell adhesion and biofilm formation, we initially introduced the pBAD::dgcZ construct into individual mutant strains. This allowed us to establish a background characterized by increased c-di-GMP levels (**Fig. 3B** [↗](#)). Subsequently, persister counting assays were performed by exposing exponentially growing transformed strains to the antibiotic ampicillin at a concentration of 150 $\mu\text{g/ml}$ for a duration of 3 hours, followed by plating. This experimental design allowed us to assess the impact of upregulated c-di-GMP on persister cell formation within specific genetic backgrounds. Results show that knocking out the toxin gene *hipH* significantly reduces the frequency of persister formation in the presence of high levels of c-di-GMP (**Fig. 3C** [↗](#)). Meanwhile, the knockout of *hipH* alone does not lead to a significant alteration in persister frequency during the exponentially growing phase. These observations suggest that HipH acts downstream of c-di-GMP signaling in the process of persister formation and antibiotic tolerance. Considering HipH belongs to the family of HipA-like Ser/Thr kinases, we investigated the

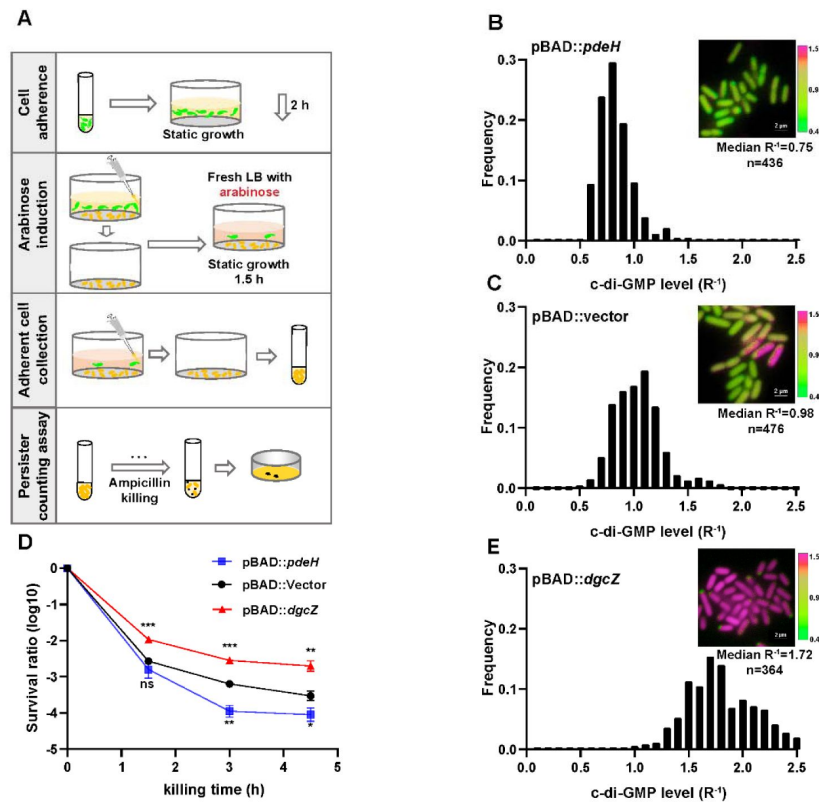


Figure 2.

High c-di-GMP levels promote persister formation.

(A) Schematic overview illustrating the manipulation of c-di-GMP levels in surface-adherent cell subpopulations for persister ratio determination. (B-C, E) Frequency distribution of c-di-GMP levels (R^{-1} score) within the surface-adherent subpopulation from strains overexpressing either PdeH (pBAD::pdeH) (B) or DgcZ (pBAD::dgcZ) (E), along with a vector control (pBAD::vector) (C). Cells preparation followed the procedure depicted in (A). Insert fluorescent images provide representative illustrations of c-di-GMP levels in adherent cells, measured using the c-di-GMP sensor system (Fig. S1B) integrated into different stains. Scale bar, 2 μ m. (D) Biphasic killing curves of the surface-adherent subpopulations from (A) ($n=3$). Error bars represent standard deviations of biological replicates. Significance was ascertained by two-tailed Student's t test. Statistical significance is denoted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

impact of knocking out *hipA*. In contrast, knocking out *hipA* had little effect on the frequency of persister formation with elevated c-di-GMP levels (Fig. 3C). This observation suggests that HipH plays a distinct role from HipA in inducing persister formation.

Given that c-di-GMP exhibits upregulation in cell-surface adhesion, we conducted a *hipH* knockout in UPEC16 strain (UPEC16- Δ *hipH*) and evaluated its influence on persister formation in biofilms (Fig. 3D). In *in vitro* biofilms, we observed a substantial decrease in persister levels following *hipH* knockout, contrasting with the wild-type strain that consistently displayed notably high levels of persister cells (Fig. 3E). These results suggest that c-di-GMP regulated HipH toxicity plays a crucial role in persister formation of biofilms. For *in vivo* biofilm formation, we introduced both the wild-type and UPEC16- Δ *hipH* strains, each at a concentration of 2×10^8 CFU, into the mouse bladders through transurethral catheterization (Pang et al, 2022) (Fig. 3D). Following 24 hours duration of infection, both strains exhibited comparable levels of intracellular bacterial communities (IBC) within the epithelial cells lining the bladder lumen (Fig. S2A-B). Subsequently, the infected mice were sacrificed, and their bladders were isolated for colony counting assays and antibiotic killing assays. Consistent with the findings from *in vitro* biofilms, the wild-type UPEC16 strain displayed a notably high rate of persister formation. In contrast, the UPEC16- Δ *hipH* strain exhibited a substantial reduction in persister cell frequency under the same conditions (Fig. 3F). The impact of HipH knockout on persister formation is consistently observed in both *in vitro* and *in vivo* settings associated with biofilms, but not in planktonic populations (Fig. 3C, left panel). These results reinforce HipH toxicity is under the regulation of c-di-GMP, and emphasize the significance of HipH in shaping the persistence and antibiotic tolerance characteristics of biofilms.

We observed a significant elevation in *hipH* gene expression upon the overexpression of DgcZ (pBAD::*dgcZ*) (Fig. 3G-H). Conversely, the strain overexpressing DgcZ-m, which is unable to increase c-di-GMP levels after induction, exhibit no activation of *hipH* gene expression (Fig. 3G). Furthermore, we labeled HipH with BFP at the C-terminal end and placed it under the *hipH* native promoter in the UPEC16 strain with c-di-GMP sensing system. Fluorescent imaging revealed a positive correlation between HipH and c-di-GMP expression at the single-cell level (Fig. S3A), supported by a correlation coefficient of 0.694 (Fig. S3B). Additionally, we engineered an *E. coli* strain in which *hipH* promoter is replaced by the constitutive promoter p-rhaB (Fig. S3C). In this strain, *hipH* expression remains unaffected by changes in c-di-GMP levels (Fig. S3D), indicating that regulation occurs at the promoter region. Furthermore, the persister ratio was comparable to that of the Δ *hipH* strain but significantly lower than that of wild-type strain under high c-di-GMP levels (Fig. S3E). These results together suggest that c-di-GMP exerts a transcriptional regulatory role in controlling the expression of HipH.

c-di-GMP and HipH form a TA-like module

We then independently overexpressed HipH (pBAD::*hipH*) to observe its impact on cell growth. The results demonstrated that HipH overexpression substantially inhibit cell growth, with only $9.8 \pm 3.4\%$ of cells retaining growth potential (Fig. 3I). Further analysis of the growth-inhibited cells revealed a predominant presence of Viable but Non-Culturable (VBNC) cells (Ayrapetyan et al, 2018; Liu et al, 2023; Pan & Ren, 2022), constituting $72 \pm 5\%$ of the total cell population, alongside a significant proportion of dead cells, accounting for $17.9 \pm 2.3\%$ of the total cells (Fig. 3J). This evidence suggests that HipH functions as a potent toxin capable of inhibiting cell growth or even inducing cell death. Interestingly, despite the observed decrease in growth potential, the persister rate remains low as that of wild-type strains (Fig. 3K). This phenomenon is interpreted as cells entering a state of deep dormancy with reduced resuscitation capabilities (Fig. 3L).

To test the role of c-di-GMP as an antitoxin in alleviating the toxic effect of HipH, we elevated intracellular c-di-GMP levels by overexpressing DosC, a moderately efficient diguanylate cyclase, or DgcZ, a highly efficient diguanylate cyclase, in the strain overexpressing HipH. This

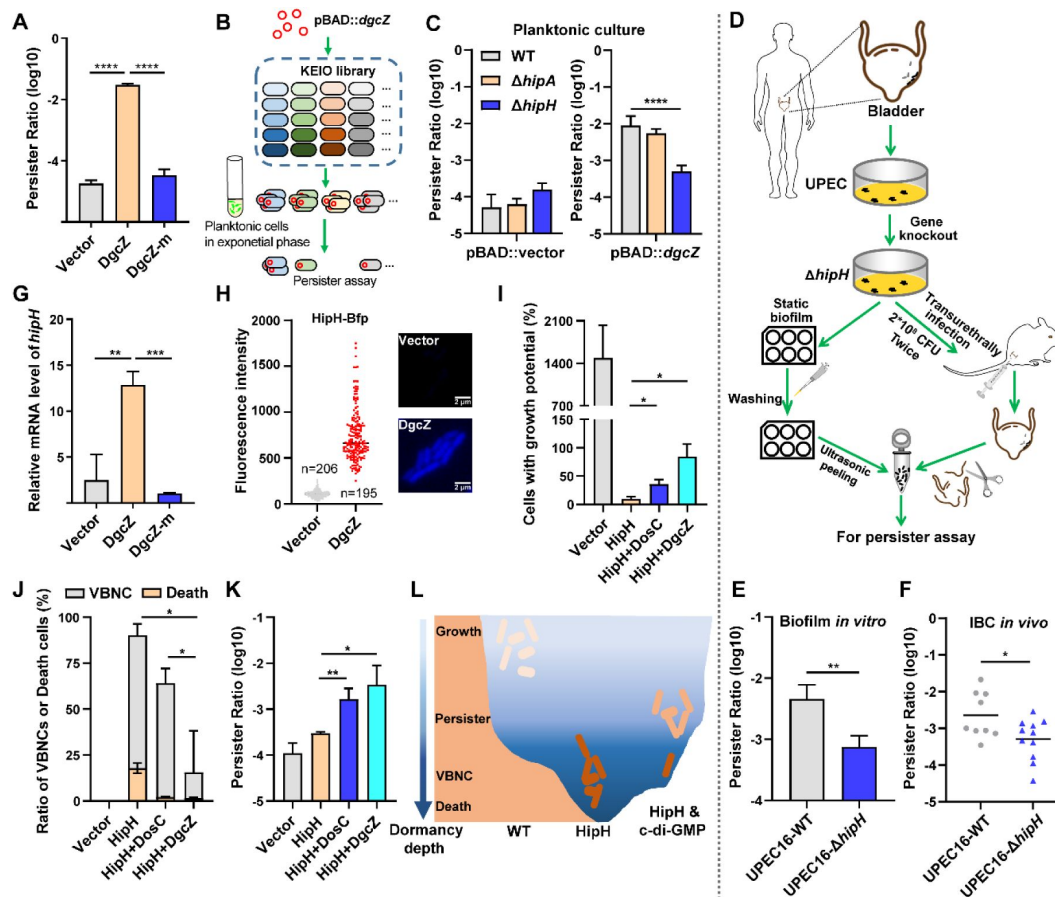


Figure 3.

HipH and c-di-GMP as a TA-like system.

(A) Determination of persister ratios in MG1655 strains overexpressing DgcZ or DgcZ-m (G206A, G207A, E208Q), with 0.002% arabinose induction for 2 h in the pBAD plasmid. Persister counting was performed following antibiotic killing (ampicillin, 150 μ g/ml, 3-hour at 37°C with shaking at 220 rpm) (n=3). (B) Schematic overview illustrating the screening of downstream targets of c-di-GMP by using *E. coli* Keio Knockout Collection, the screen was conducted in the presence of high levels of c-di-GMP by inducing the expression of DgcZ with 0.002% arabinose for 2 h. (C) Identification of HipH as the downstream target of c-di-GMP in persister formation in Keio strains. Left panel: persister assay performed without high levels of c-di-GMP (pBAD-vector); Right panel: persister assay performed in the presence of high levels of c-di-GMP (pBAD-dgcZ) (ampicillin, 150 μ g/ml, 3-hour at 37°C with shaking at 220 rpm) (n=9). (D) Schematic overview of clinical UPEC16 isolation, genetic modification, and *in vitro* or *in vivo* biofilm culture. (E) Antibiotic killing (ampicillin, 150 μ g/ml, 3-hour at 37°C with shaking at 220 rpm) and persister counting assay for *in vitro* biofilm cells (n=3). (F) Antibiotic killing (ampicillin, 150 μ g/ml, 3-hour at 37°C with shaking at 220 rpm) and persister counting assay for *in vivo* IBC cells (n=9 for WT, n=11 for Δ hipH). (G) Relative expression level of *hipH* in MG1655 strains under different levels of cellular c-di-GMP, determined by RT-qPCR and normalized to the levels of 16S rRNA. DgcZ and DgcZ-m were induced by 0.002% arabinose for 2 h in the pBAD plasmid (n=3). (H) Fluorescence intensity of HipH fused to BFP in MG1655 strains under varying levels of cellular c-di-GMP. DgcZ was induced by 0.002% arabinose for 2 hours using the pBAD plasmid. The inserted fluorescent images provide representative illustrations of HipH-BFP. (I-K) Detection of the ratio of cells with growth potential (I), VBNCs or death cells (J) and persisters (K) in MG1655 strains overexpressing HipH alone, or overexpressing HipH and DosC together, or overexpressing HipH and DgcZ together, induced by 0.002% arabinose for 4 hours. Persister counting performed followed antibiotic killing (ampicillin, 150 μ g/ml, 3-hour at 37°C with shaking at 220 rpm) (n=3). (L) Schematic representation of the dormancy depth controlled by HipH and c-di-GMP, and the relationship with cell state. Error bars represent standard deviations of biological replicates. Significance was ascertained by two-tailed Student's t test (ACEFGHK) or Two-way ANOVA (I-J). Statistical significance is denoted as *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001.

manipulation resulted in a 2.72-fold and 4.22-fold increase in the mean of c-di-GMP R^{-1} score, respectively (Fig. S3F). Comparing to the strain overexpressing HipH alone, those strains co-overexpressing HipH and c-di-GMP exhibited a significantly recovered growth potential (Fig. 3I). Specifically, $36 \pm 6.7\%$ of cells retained growth potential in the strain expressing HipH and DosC, while approximately $85 \pm 18\%$ retained growth potential in the strain expressing HipH and DgcZ. Further analyzing the portion of growth-inhibited cells revealed that the strain overexpressing HipH and DosC exhibited a decreased rate of VBNC cells ($62 \pm 6\%$ of the total cell population) and dead cells ($2.1 \pm 0.2\%$ of the total cell population) (Fig. 3J). The strain overexpressing HipH and DgcZ showed an even lower rate of VBNCs ($14 \pm 17\%$ of the total cell population) and dead cells ($1.5 \pm 0.4\%$ of the total cell population) (Fig. 3J). These results support the assumption that c-di-GMP acts as an effective antitoxin, enabling HipH-toxified cells to recover their growth potential. Consistently, increased rates of persister cells were observed, with $10^{-2.8 \pm 0.19}$ in the strain overexpressing HipH and DosC, and $10^{-2.5 \pm 0.34}$ in the strain overexpressing HipH and DgcZ (Fig. 3K). These results collectively indicate that c-di-GMP functions as an antitoxin in alleviating HipH's harmful effects, steering cells from deep dormancy depth to a shallower dormancy state with enhanced resuscitation capabilities, thereby promoting persister cell formation (Fig. 3L).

HipH is a deoxyribonuclease inducing genome instability

hipH stands out as an atypical toxin gene due to its lack of a genetically linked antitoxin cognate, existing as a solitary gene unlike the typical toxin-antitoxin gene pairs found within the same operon (Jurenas et al, 2022; Maeda et al, 2017). Previous studies believed it a homolog of the toxin protein HipA, functioning as a Ser/Thr protein kinase involved in regulating ribosome assembly and cell metabolism (Gratani et al, 2023). However, our findings challenge this prevailing belief. The truncated mutant of HipH, lacking the kinase catalytic motif ($\Delta 5$, amino acid 340-372), demonstrated a comparable ability to inhibit cell growth and induced VBNCs compared to the intact HipH (Fig. 4A-C). These results suggest the involvement of another domain in HipH that is responsible for its toxic activity.

Structure analysis revealed that, in addition to its kinase catalytic motif (amino acid 340-372), HipH also contains a DNA binding HTH domain (amino acid 11-32), a gly loop and autophosphorylation site (amino acid 190-215), and an activation loop (amino acid 222-229) (Fig. 4A). To identify the specific domain responsible for its toxicity, we generated various truncated mutants and evaluated their capacity in inhibiting cell growth. Our results showed that overexpression of mutants lacking the DNA binding domain HTH ($\Delta 1$ and $\Delta 2$) did not inhibit cell growth or induce VBNCs or dead cells (Fig. 4B-C). This indicates that the DNA binding domain HTH is indispensable for the observed toxicity of HipH. Consistent with these results, fluorescent microscopy confirmed the nucleoid localization of wild-type HipH (Fig. 4D), indicating that the primary function of HipH is related to DNA interaction.

We observed the elongated filamentous shape induced by HipH overexpression in cells (Fig. S4A), which is consistent with previous findings (Gratani et al, 2023). We interpret this as a potential characteristic associated with DNA damage. Furthermore, considering the results of the truncated mutation assay (Fig. 4A-C), we hypothesize that HipH plays a role in inducing DNA damage. To investigate this hypothesis, we first examined whether HipH overexpression induces the SOS response, a bacterial defense mechanism against DNA damage involving the expression of various genes. qPCR analysis revealed a significant upregulation of SOS response genes, such as *recA*, *sulA*, and *tisB*, in cells overexpressing HipH (Fig. S4B). Notably, the near abolition of cell growth potential upon knockout of *recA*, a crucial protein essential for DNA damage repair, in cells overexpressing HipH further supports the conclusion that HipH induces DNA damage (Fig. S4C).

To investigate the specific type of DNA damage caused by HipH, we conducted *in vitro* DNA-cleaving assays (Hockings & Maxwell, 2002; Maki et al, 1996) using purified HipH. To address its insolubility, we engineered a fusion construct with a high solubility protein, GFP, at the C-

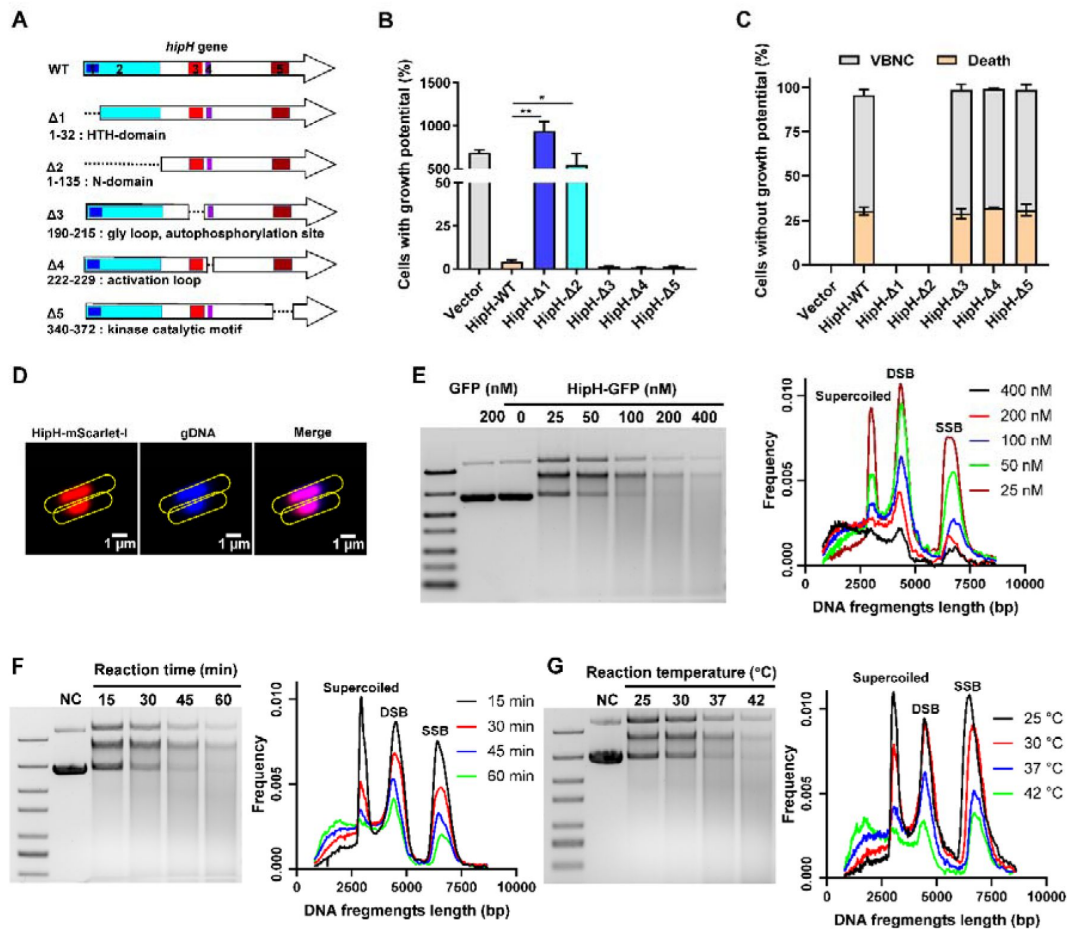


Figure 4.

HipH is a deoxyribonuclease exerting genotoxic effects.

(A) Structure analysis of HipH protein and Schematic representation of truncated mutants illustrating key structural domains. (B-C) Detection of the ratio of cells with growth potential (B) and VBNCs or death cells (C) in MG1655 strains overexpressing different truncated HipH mutants, induced by 0.002% arabinose for 3 h (n=3). (D) Subcellular localization of HipH in MG1655 strain. HipH was fused with mScarlet-I and induced by 0.002% arabinose for 2 hours. The gDNA was stained with Hoechst 33258 for 15 minutes at room temperature. Scale bar, 1 μm. (E-G) Left panel: *in vitro* DNA cleavage assay using 500 ng supercoiled plasmid DNA, (E) with varying concentrations of HipH-GFP (0, 25, 50, 100, 200 or 400 nM), with purified GFP protein as control; (F) with different durations of reaction time (15, 30, 45 and 60 minutes); (G) with different reaction temperature (25, 30, 37 and 42°C). If not specified, 200 nM of HipH-GFP was added to each reaction. "NC", negative control, indicates supercoiled plasmid without addition of HipH-GFP. Right panel: illustration of the relationship between the length of DNA fragments and their ratio to total DNA, as indicated in the corresponding upper panel. SSB, single-strand break; DSB, double strand break. Error bars represent standard deviations of biological replicates. Significance was ascertained by Two-way ANOVA. Statistical significance is denoted as *P < 0.05, **P < 0.01.

terminal end to facilitate protein purification. The results demonstrated HipH's capability to cleave supercoiled plasmids, leading to the production of double-strand breaks (DSBs), as evidenced by prominent smears observed on agarose gels (**Fig. 4E-G**). Further analysis revealed a positive correlation between HipH's DNA cleavage capacity and its protein concentration, temperature, and incubation time (**Fig. 4E-G**). These findings collectively indicate that HipH cleaves DNA and produces DSBs. The overexpression of RecG, a key player in the repair of DSBs in DNA (Whitby et al, 1994), successfully rescued growth deficient cells resulting from HipH overexpression (**Fig. S4D**), providing additional evidence that HipH induces DSBs in DNA. These compelling results demonstrate that HipH functions as a deoxyribonuclease, exerting genotoxic effects on genomic DNA integrity.

c-di-GMP acts as an antitoxin to alleviate HipH genotoxicity

To investigate whether c-di-GMP plays as an antitoxin to mitigate HipH genotoxicity, we first explored the potential of direct interaction between c-di-GMP and HipH. A biotinylated c-di-GMP pull-down assay (**Fig. 5A**) (Chambers & Sauer, 2017), conducted from both cell lysate and purified protein, revealed a physical binding between c-di-GMP and HipH (**Fig. 5B-D**, **Fig. S5A-B**), confirming their direct interaction. The dissociation constant (K_D), calculated based on the concentration of c-di-GMP at which half-maximal binding of HipH occurs, was determined as 7.6 μ M (**Fig. 5B**, bottom panel). Additionally, c-di-GMP successfully displaced HipH-GFP-His from HipH-GFP-His/biotin-c-di-GMP complexes, while other nucleotides such as cAMP and GTP did not exhibit the same effect (**Fig. 5E**). This underscores the specificity of the binding between c-di-GMP and HipH.

Subsequently, we introduced c-di-GMP into the HipH DNA-cleavage assay system, and notably, its addition exerted a significant suppressive effect on the cleavage activity of HipH. This effect was observed for both relaxed and supercoiled plasmids, as evidenced by a reduction of smears and double-strand break (DSB) bands on agarose gels (**Fig. 5F-I**). It is noteworthy that other nucleotides, including cAMP and GTP, did not demonstrate the same suppressive effect on HipH's cleavage activity (**Fig. S5C**). Furthermore, it has been reported that HipB serves as an antitoxin for HipH (Gratani et al, 2023; Maeda et al, 2017). However, when we tested the potential impact of HipB on HipH's DNase activity by purifying and introducing HipB into our cleavage system, we found that HipB failed to inhibit HipH's DNase activity (**Fig. S5C**). These findings suggest that c-di-GMP specifically interacts with HipH, leading to the inhibition of its DNase activity.

To monitor DNA breakage in live bacterial cells with varying stoichiometry between HipH and c-di-GMP, we utilized Gam-GFP, a double-strand DNA end-binding protein of bacteriophage Mu that forms foci at DSBs in living cells (Shee et al, 2013). Microscopic examination revealed that cells overexpressing HipH alone displayed Gam-GFP foci in $8.85 \pm 1.28\%$ of cases, while only $0.30 \pm 0.12\%$ of wild-type cells demonstrated such foci (**Fig. 5J-K**). Further investigations showed that elevation of intracellular c-di-GMP levels resulted in a significant reduction in the percentage of cells with Gam-GFP foci, with DosC addition decreasing the occurrence to $1.34 \pm 0.39\%$ and DgcZ to $0.46 \pm 0.19\%$ (**Fig. 5J-K**). These *in vitro* and *in vivo* findings conclusively demonstrate that c-di-GMP acts as an antitoxin for HipH, effectively suppressing its genotoxic effects.

Discussion

In the intricate biofilm dynamics, the origin of persister formation has largely been ascribed to the constraints imposed by the dense structures, such as limited nutrients, oxygen, and antibiotics penetration, or quorum sensing. Our investigation reveals that cell adhesion, a pivotal event initiating biofilm development, emerges as a trigger for persister formation. This concept introduces a layer of complexity that reshapes our understanding of drug tolerance in biofilms.

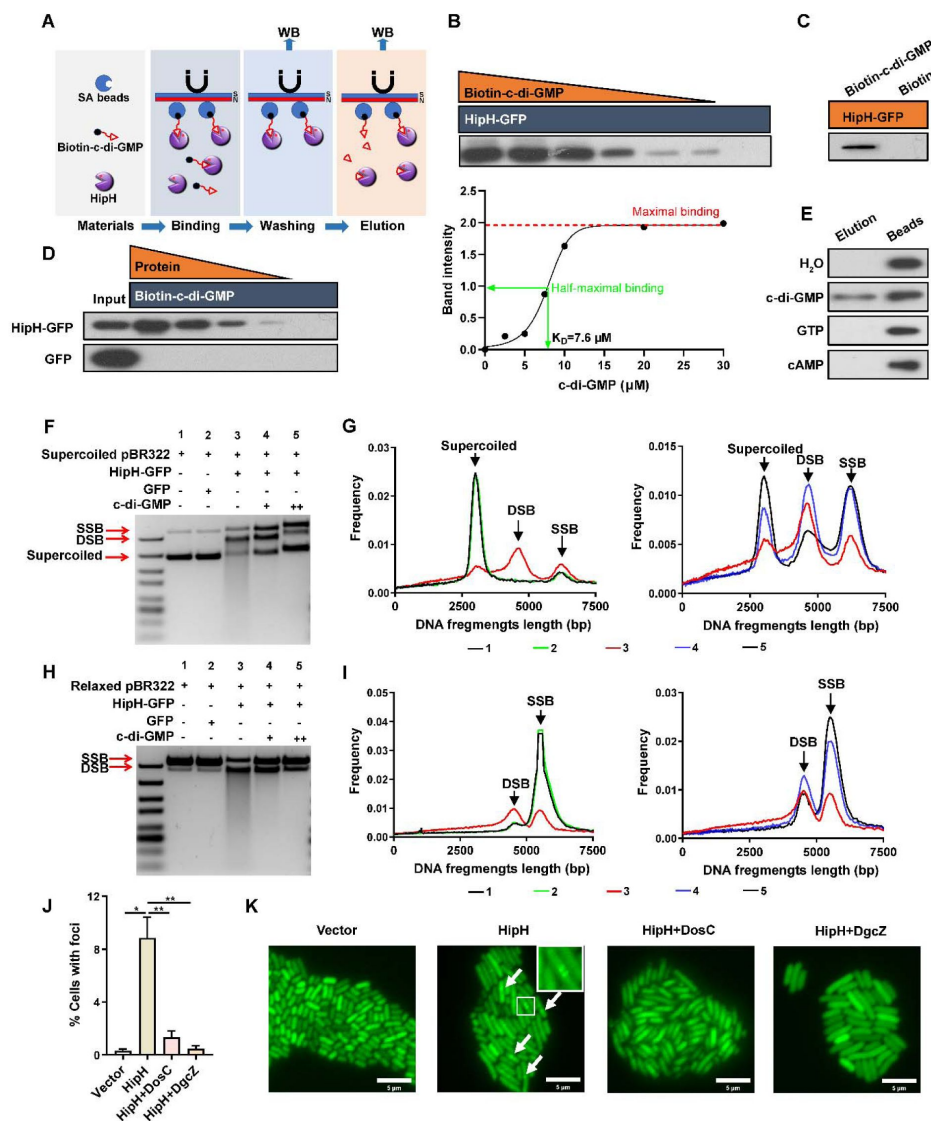


Figure 5.

c-di-GMP acts as an antitoxin to repress HipH DNA Cleavage Activity.

(A) Schematic representation of the biotin-c-di-GMP pull-down assay for HipH protein. (B-E) Identification of the interaction between c-di-GMP and HipH using the biotinylated c-di-GMP pull-down assay. (B) Upper panel: 1.5 μ M purified HipH-GFP-His and biotin-c-di-GMP were added in gradient concentrations (30, 20, 10, 7.5, 5, 2.5, 0 μ M). Bottom panel: the intensity of bound protein, obtained by analyzing the immunoblot shown in the upper panel using ImageJ, relative to the concentrations of c-di-GMP used in the pull-down assays. (C) 0.3 μ M purified HipH-GFP-His and 20 μ M biotin-c-di-GMP or biotin were added. (D) Purified HipH-GFP-His or GFP-His was added in gradient concentrations (1.5, 0.75, 0.375, 0.19, 0 μ M), and 20 μ M biotin-c-di-GMP was added. 0.5 μ g protein was used as the input sample. (E) 2 mM c-di-GMP, cAMP, or GTP were used to elute HipH-GFP-His from SA beads, as well as HipH-GFP-His/biotin-c-di-GMP complexes prepared from the pull-down reaction mix containing 1.5 μ M purified HipH-GFP-His and 20 μ M biotin-c-di-GMP. (F-I) *In vitro* DNA cleavage assay using 500 ng supercoiled plasmid DNA (F-G) or relaxed plasmid DNA (H-I). "+" indicated 1.67 mM c-di-GMP and "++" indicated 5 mM c-di-GMP. 200 nM HipH-GFP-His or GFP-His was used in Cleavage reaction at 37°C for 1 h. (G) and (I) represent the relationship between the length of DNA fragments and their ratio to total DNA, as shown in B and D, respectively. SSB: single-strand break (relaxed plasmid DNA); DSB: double-strand break. (J) Statistic analysis of the ratio of cells with Gam-GFP foci in different MG1655 strains: overexpressing HipH alone, or HipH with DosC, or HipH with DgcZ (n=3). (K) Representative images of cells expressing Gam-GFP in the aforementioned different strains. Scale bar, 5 μ m. Error bars represent standard deviations of biological replicates. Significance was ascertained by Two-way ANOVA. Statistical significance is denoted as *P < 0.05, **P < 0.01.

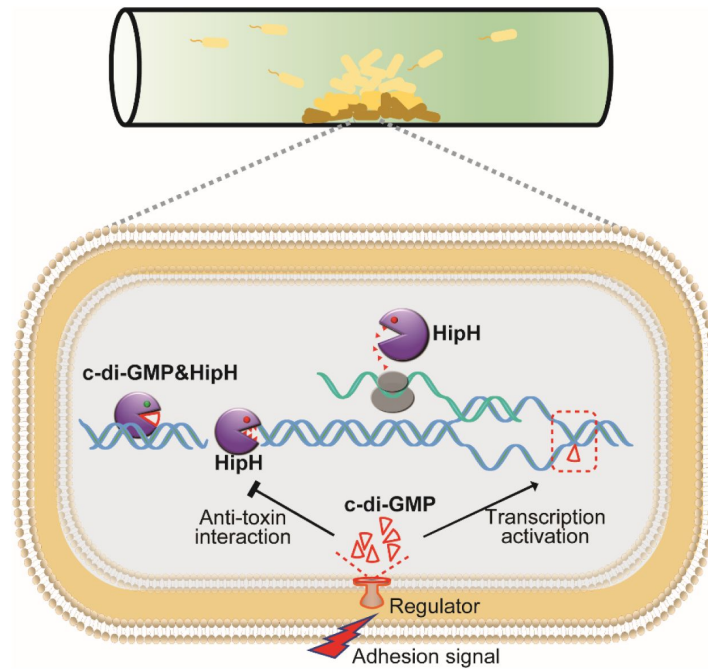


Figure 6.

Schematic overview of the mechanism underlying c-di-GMP induced persister formation in biofilms.

In biofilms, cell-surface adhesion triggers an increase of intracellular c-di-GMP levels. Elevated intracellular c-di-GMP levels inducing the expression of HipH. The HipH protein is a deoxyribonuclease which induces DNA double-strand break. Acting as an antitoxin, c-di-GMP mitigates the genotoxicity of HipH.

Beyond the traditional emphasis on dense biofilm structures, the scope should now encompass single-layer cells on the surface. Such a deviation from conventional perspectives highlights the necessity for a more nuanced and comprehensive approach to address the challenges of antibiotic efficacy within biofilm communities.

Mechanism Linking Surface Adhesion to Persister Cell Formation in Bacterial Biofilms

This study provides novel insights into the relationship between bacterial adhesion to surfaces and the subsequent increase in persister cell formation, which has not been explicitly detailed in previous literature. While existing research has established that biofilms typically harbor higher numbers of persister cells, this investigation not only corroborates that finding but also elucidates the mechanisms through which surface adhesion contributes to this phenomenon. Past studies have predominantly focused on the general characteristics of persister cells and their role in biofilm resilience and antibiotic tolerance without specifically addressing the mechanistic link between adhesion and persister formation (Pan et al, 2023 [↗](#); Patel et al, 2022 [↗](#)). For instance, previous work has shown that surface attachment leads to changes in metabolic activity and signaling pathways within bacterial cells, which could promote persistence, but it has not definitively established a causal relationship between adhesion and increased persister formation. Our study highlights that the elevation of c-di-GMP levels after surface adhesion triggers a cascade of physiological changes that significantly enhance the formation of persister cells. In particular, we report that adhesion-induced signaling pathways promote dormancy and tolerance to antibiotics, marking an important advancement from the previous understanding that treated persister cells might arise from random phenotypic variation during biofilm development.

Noncanonical Toxin-Antitoxin Systems involving with small metabolic molecules?

c-di-GMP emerges as a pivotal secondary messenger in the intricate orchestration of bacterial lifestyles, playing a key role in mediating cell-surface adhesion and exerting influence over the transition from planktonic to sessile existence. Our comprehensive exploration, employing a spectrum of techniques, brings to light a significant revelation: heightened levels of c-di-GMP in cells drive the formation of persisters. Notably, c-di-GMP functions as a noncanonical antitoxin, regulating both the expression and genotoxicity of the toxin protein HipH. This small molecule, composed of just two guanine bases, counteracts the toxin's effects.

Another intriguing parallel to our findings can be drawn from the ppGpp-SpoT system, which exemplifies how small metabolic molecules can function in a manner reminiscent of traditional toxin-antitoxin (TA) systems (Amato et al, 2013 [↗](#)). At the core of this system is the synthesis and hydrolysis of ppGpp (guanosine 5'-diphosphate 3'-diphosphate), modulating gene expression for stress adaptation. In this TA-like paradigm, ppGpp assumes the role of the "toxin", while SpoT functions as the "antitoxin". SpoT exhibits dual functionality, both synthesizing and degrading ppGpp in response to various stresses, thereby regulating the levels of this potent signaling molecule.

Both systems play crucial roles in bacterial stress adaptation and persistence regulation. The ppGpp-SpoT system employs ppGpp as a toxin-like molecule coordinating various physiological processes. In contrast, the HipH-c-di-GMP interaction involves c-di-GMP as an antitoxin-like molecule influencing persister cell formation by regulating HipH, a genome instability-inducing toxin. These systems expand the conceptual framework of TA systems. While traditional TA systems consist of genetically linked gene pairs encoding a toxin and its neutralizing antitoxin (Jurenas et al, 2022 [↗](#); Qiu et al, 2022 [↗](#); Srivastava et al, 2021 [↗](#)), these new systems introduce additional complexities and nuances to our understanding of bacterial regulatory mechanisms. The involvement of metabolites like ppGpp and c-di-GMP in TA-like systems demonstrates the

intricate interplay between cellular metabolism and stress response pathways. This integration allows bacteria to fine-tune their responses to environmental challenges with remarkable precision. Moreover, these systems underscore the evolutionary adaptability of bacteria, showcasing how they have co-opted metabolic molecules into sophisticated regulatory networks.

Novel functions of toxin protein HipH

HipH emerges as an intriguing toxin within the bacterial realm, standing apart due to its solitary existence without a genetically linked antitoxin cognate—an uncommon trait in known toxin-antitoxin systems. Previous studies have identified HipH as a Ser/Thr protein kinase, sharing homology with the well-characterized toxin HipA. These studies demonstrated its primary protein substrates to be the ribosomal protein RpmE (L31) and the carbon storage regulator CsrA (Gratani et al, 2023 [DOI](#)). While the overproduction of HipH was observed to impact bacterial DNA segregation and cell division, the underlying mechanism remained unexplained. In this study, we have uncovered that HipH primarily functions as a potent DNase, providing an explanation for the observed phenomenon. Our findings further reveal that HipH, as a potent DNase, induces a deep cell dormancy depth and elevated rates of VBNC cells and dead cells, rather than persister cells with the capacity of resuscitation. Significantly, our investigation further sheds light on the regulation of HipH genotoxicity by c-di-GMP in a dual manner. Firstly, high intracellular levels of c-di-GMP induce HipH expression. Subsequently, c-di-GMP acts as an antitoxin, mitigating the potent toxicity of HipH. This, in turn, maintains cells with an adequate dormancy depth, facilitating the formation of persister cells and conferring antibiotic tolerance. It is intriguing to delve deeper into research exploring the domain-function relationship in HipH. However, our efforts have often encountered challenges, as mutations can readily induce protein aggregates, impeding thorough analysis. Achieving a comprehensive understanding of this domain-function relationship necessitates the development of innovative methodologies and approaches.

Taken together, we reveal a sophisticated regulatory circuit underlying bacterial genome instability and antibiotic persistence in biofilms. This regulated genome instability can have significant implications for adaptive potential of bacterial populations, shaping their ability to persist, evolve, and develop resistance mechanisms. By targeting this c-di-GMP regulated genotoxicity of toxin protein HipH, we successfully inhibited drug tolerance in UPEC induced biofilms. These findings provide potential therapeutic strategies for combating chronic and relapsing infections associated with biofilms.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study included *Escherichia coli* strains MG1655, BW25113, UPEC16, and the Keio library Knockout Collection (**Supplementary Table S1** [DOI](#)). UPEC16 was isolated from a urine sample (#16) obtained from a local urinary tract infection (UTI) patient at the First Affiliated Hospital of Kunming Medical University, following the guidelines of the Committee on Human Subject Research and Ethics (2022-L-203 and CHSRE2022030). The genome sequence has been deposited in the National Genomics Data Center under the accession number "GWHERG100000000". All strains were cultured in Luria Broth (LB) medium, with routine growth condition at 37°C and 220 rpm. To support plasmid maintenance, media were supplemented with the corresponding antibiotics as needed: chloramphenicol (25 µg/ml), kanamycin (20 µg/ml), gentamicin (20 µg/ml), and ampicillin (100 µg/ml). For inducible expression experiments, the medium was supplemented with inducers such as 0.002%-0.2% arabinose for the Arabinose-induction system, 1 mM IPTG for the Lac-induction system, and 1 µg/ml anhydrotetracycline for the Tet-induction system.

Strains construction

Construction of Target DNA Knockout

The generation of target DNA knockout strains involved the use of λ -red mediated gene replacement (Datsenko & Wanner, 2000 [\[1\]](#)). In this procedure, a 500 bp upstream region of the target DNA sequence, a resistance cassette for substitution (CmR for UPEC16 Δ *hipH*, GmR for UPEC16 Δ *araD-B*), and a 500 bp downstream region of the target DNA sequence were fused through fusion PCR. The resulting fusion fragments were then transformed into electrocompetent cells along with a recombineering helper plasmid, pSim6 (Datta et al, 2006 [\[2\]](#)). After a recovery period of 3-5 hours, the transformed cells were plated on selection plates containing the corresponding antibiotics. The antibiotic-selected recombinant colonies were subsequently validated through PCR analysis and sequencing. Finally, the correct recombinants were incubated at 42°C to eliminate the temperature-sensitive helper plasmid pSim6.

Recombinant Plasmid Construction

Construction of recombinant plasmids was performed using the 2 × MultiF Seamless Assembly Mix (ABclonal, RK21020). To tag HipH, *hipH*, together with its native promoter, was fused with Blue Fluorescent Protein gene *bfp* and inserted into the pBAD backbone by *in vitro* recombination. For Gam-GFP overexpression, the *gam* gene from bacteriophage Mu was fused with Green Fluorescent Protein gene *gfp* and inserted into a p15A ori plasmid by *in vitro* recombination. The *gam-gfp* was placed under the control of the Tetracycline induction system (Addgene, pFD152, #125546). Similarly, for *recG* overexpression, the *recG* gene was cloned into a p15A ori plasmid, placed under the control of the T5 promoter-Lac-induction system. To express HipH-His, mCherry-His, DosC, and DgcZ (DgcZ-m), these genes were cloned into pBAD, under the control of the Arabinose-induction promoter. Additionally, for the c-di-GMP sensor system purchased from Addgene (#182291), the plasmid origin was substituted with the p15A ori.

Static biofilm culture

The methodology employed in this study was adapted from the established one (Merritt et al, 2005 [\[3\]](#)). Bacterial strains of interest were initially inoculated in a 3-to-5-ml culture in tubes and allowed to grow overnight. Subsequently, the stationary cultures were diluted 1:100 in the desired media and cultured until reaching the exponential phase with an OD600 of 0.3. Next, 2 ml of each diluted culture was transferred into a fresh microtiter dish (Biosharp, Cat#BS-20-GJM), which has not been undergone tissue culture treatment. In cases where the subsequent experiment involves imaging, it is necessary to ensure that the microtiter dish has a glass bottom. The plate was covered and incubated at optimal growth temperature for the desired amount of time. While the lids for the microtiter dishes are reusable, it is important to clean them with 70% (v/v) ethanol and allow them to air-dry prior to each experiment.

For cultures involving PdeH or DgcZ induction, it is important to statically incubate cells in microtiter dish for 2 hours at 37°C first. This incubation period allows cells adherence to the surface. Subsequently, the inducer arabinose was added to the desired concentration to initiate protein induction with desired duration.

Antibiotic killing assay and persister counting

For cells from static culture, planktonic cells were directly transferred into new tubes and harvested by centrifugation. The remained microtiter dishes were gently washed twice by PBS buffer, and adherent cells were harvested by vigorous pipetting. In other cases, the cells were harvested routinely.

Subsequently, the harvested cells directly plated on LB plate for colony-forming unit (CFU) counting without antibiotic challenge.

The harvested cells were washed with PBS buffer and subsequently resuspended into fresh LB containing 150 µg/ml ampicillin and incubated for varying durations (0 to 4.5 hours) at 37°C with shaking at 220 rpm. The survived cells after ampicillin challenge were harvested by centrifugation and diluted in PBS, followed by plating on LB plate for overnight culture at 37°C. Persister counting was performed on the next day. Persister ratios were calculated as the ratio of the CFU after antibiotic treatment to the CFU without antibiotics challenge. At least three biological replicates were performed for each experiment. Averages and standard deviations presented are representative of biological replicates.

Cell growth potential assay and VBNC determination

To evaluate growth potential, bacterial cells were cultured to exponential phase in LB medium to an OD600 of 0.3. For strains harboring an arabinose-induction system for specific protein expression, 0.002% arabinose (w/v) was added to the medium for 3 to 4 hours at 37°C with shaking at 220 rpm. The cells were then plated for colony-forming unit (CFU) counting on LB plates before and after arabinose induction. The cells with growth potential were determined as the ratio of CFUs after arabinose induction to CFUs before arabinose induction. The dead cells ratio was assessed by staining with PI dye. The ratio of VBNC cells was calculated as 100% minus the sum of the ratios of cells with growth potential and dead cells. Averages and standard deviations presented are representative of three biological replicates.

Microscopy

Bright-field and fluorescence imaging

Inverted microscope Nikon (eclipse Ti2) and confocal microscope (Leica, Stellaris 5 WLL) were used in this study. The illumination was provided by different lasers, at wavelengths 405 nm for BFP and Hoechst 33258, 488 nm for GFP and SYTOTM24, 488 nm (Nikon) or 515 nm (Leica) for mVenus^{NB}, 561 nm for propidium iodide (PI) and mScarlet-I, respectively. The fluorescence emission signal was imaged to an sCMOS camera (pco.edge 4.2 bi). Appropriate filter sets were selected for each fluorophore according to their spectrum. Image analysis was done by ImageJ software (Fiji). For c-di-GMP sensor analysis, the ratio of mVenus^{NB} to mScarlet-I (R) has a negative correlation with the concentration of c-di-GMP. Therefore, R^{-1} demonstrates a positive correlation with the concentration of c-di-GMP.

Cells staining

PI, Hoechst 33258 and SYTOTM24 were added to culture at a final concentration of 1 µg/ml, 10 µg/ml and 10 µM, respectively. The cells were then incubated in the dark at room temperature for 15 to 30 minutes. Prior to imaging, the cells were washed twice with PBS.

Gam-GFP foci imaging

The method employed in this study was adapted from the established one (Shee et al, 2013 [\[1\]](#)). Cells expressing toxin protein HipH along with DosC or DgcZ under the arabinose-induction system and expressing Gam-GFP under Tetracycline-induction system were cultured to exponential phase in LB medium with supplement of adequate antibiotics for plasmid maintenance. Gam-GFP was induced by 1 µg/ml anhydrotetracycline for 30 minutes and then HipH and DosC (or DgcZ) were induced by 0.2% (w/v) arabinose for 2.5 h at 37°C and 220 rpm. The cells were washed twice using PBS buffer and then visualized under a fluorescence microscope. The Gam-GFP foci were analyzed using ImageJ software from the individual cell intensity histograms.

At least four neighboring pixels, each with an individual fluorescence intensity (f) greater than 1.2 times the mean fluorescence intensity of the cell (a), were deemed as foci. Meanwhile, the area occupied by these pixels needed to be less than one-fifth of the total bacterial area.

Time-lapse imaging

For recording antibiotic killing and bacteria resuscitation processes, cells harboring c-di-GMP sensor in the 24-hour static culture were collected and washed twice with PBS buffer and imaged on a gel-pad containing 3% low melting temperature agarose in PBS (Pu et al, 2019 [DOI](#); Pu et al, 2016 [DOI](#)). The gel-pad, prepared at the center of the FCS3 chamber as a gel island, was observed under bright field or epifluorescence illumination. Subsequently, the gel-pad was encircled by LB broth containing 150 $\mu\text{g/ml}$ ampicillin for 6 hours at 35°C. Fresh LB broth was introduced, and the growth medium was refreshed every 3 hours to allow cells to recover sufficiently.

Fluorescence-activated Cell Sorting FACS analysis

All samples were analyzed using a Beckman CytoFLEX SRT flow cytometer equipped with a 100 μm nozzle, and normal saline served as the sheath fluid. The c-di-GMP sensor-labeled strain in the 24-hour static biofilm growth phase was washed and resuspended in sterile PBS. Bacterial cells were identified based on FSC (forward scatter) and SSC (side scatter) parameters. Cells were sorted into distinct groups according to their fluorescence intensity (PB450 for BFP, FITC for mVenus^{NB}, ECD for mScarlet-I). The obtained results were analyzed using FlowJo V10 software (Treestar, Inc.).

Protein purification

All proteins used in this study were purified through affinity chromatography using a His-tag and a nickel column. Briefly, MG1655 Δara harboring pBAD::hipH-GFP-His (or GFP-His) grew to exponential growth phase in 500 ml LB medium. Induction of HipH-GFP-His (or GFP-His) was carried out by 0.002% arabinose for 2 hours at 37°C with shaking at 220 rpm. The cells were harvested and resuspended in 25 ml binding buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM imidazole, 0.5% Triton X-100, pH 8.0) containing 1 mg/ml lysozyme. The samples were incubated at 37 °C for 30 minutes and then subjected to ultrasonic disruption. The cell lysate was centrifuged at 12,000 g for 20 minutes at 4°C, and the supernatant was collected for protein purification. Ni-TED SefinoseTM Resin (Sangon Biotech, C610030) was utilized for the purification of His-tagged proteins as per the manual. Finally, HipH-GFP-His (or GFP-His) was stored in elution buffer containing 10% glycerol at -80°C.

RNA extraction and real-time quantitative PCR (RT-qPCR) assay

Bacterial strains were grown to mid-exponential growth phase and then induced by 0.002% arabinose for 2 hours. Cells were harvested and washed by PBS twice. Total RNA extraction was carried out using total RNA extraction reagent (Vazyme R403, China), following the manufacture's instruction. Subsequently, 1 μg of total RNA was digested with DNase I and reverse transcribed to cDNA using a reverse transcription kit (Vazyme R223, China). The resulting cDNA was used as template for RT-qPCR (Vazyme Q711, China). The RT-qPCR assay were analyzed using BIO-RAD CFX Connect Real-Time PCR Detection Systems. The 16S rRNA gene was selected as the internal control. The primers used in RT-qPCR assay were listed in **supplementary Table S1** [DOI](#). All experiments were performed in biological triplicates to ensure reliability and reproducibility of the results.

Biotin c-di-GMP pull down assay

The pull-down assay was conducted as previously described (Chambers & Sauer, 2017 [DOI](#)) with some modifications. In the case of using purified proteins, purified HipH-GFP-His or GFP-His protein was added into binding mix containing biotin-c-di-GMP (200 pmol/ μl , BIOLOG, B098), 0.2 μl EDTA (200 mM), 2 μl 10 $\times$ buffer (100 mM Tris-HCl, pH 7.5, 500 mM KCl, 10 mM DTT). The reaction mix was brought to a final volume of 20 μl with the addition of H₂O and then incubated at 25°C for

20 minutes. In the case of using cell lysate, MG1655 Δ *ara* harboring pBAD::*hipH*-His (or *mCherry*-His) was cultured to exponential phase with an OD600 of 0.3 in 30 ml LB medium. 0.002% arabinose was then added into the culture for another 2 h at 37°C with shaking at 220 rpm. The cells were harvested and resuspended in 1.5 ml lysis buffer (TBST containing 1 mg/ml lysozyme, 5 µg/ml DNaseI, 1× protease inhibitor), followed by incubation at 37°C for 30 minutes and three cycles of freezing and thawing. The cell lysate was centrifuged at 12,000g for 20 minutes at 4°C, and the supernatant was collected for the pull-down assay. The specified amount of cell lysate was introduced into a reaction mix containing the indicated amount of biotin-c-di-GMP, 1 µl EDTA (200 mM), and 10 µl 10× buffer. The reaction mix was brought to a final volume of 100 µl with the addition of H₂O and then incubated at 25°C for 20 minutes.

10 µl (per sample) Streptavidin (SA) magnetic beads (Thermo Scientific, 88816) was washed twice using TBST and resuspended in 150 µl TBST. Washed SA beads were added into reaction buffer and incubated at 25°C for 1 h on a rotary table. Subsequently, the SA beads were washed five times using TBST.

Biotin-c-di-GMP-conjugated proteins on SA beads were eluted with SDS loading buffer and analyzed by Western blot.

DNA cleavage assay

For DNA cleavage, 500 ng supercoiled plasmid pBR322 (Chan et al, 2015 [DOI](#)) obtained from the FastPure Plasmid Mini Kit (Vazyme, DC201-01) or relaxed plasmid pBR322 (derived from supercoiled pBR322 digested by Topoisomerase I, Takara, 2240A), along with 6 µl 5× buffer (170 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM DTT, 20 mM MgCl₂, 25 mM spermidine, 25% Glycerol) and 3 µl BSA (1 mg/ml), were added to the binding mix (as mentioned in Biotin c-di-GMP pull down assay section).

The reaction mix was brought to a final volume of 30 µl with the addition of H₂O and then incubated at 37°C for varying duration. The reactions were halted by adding SDS to a final concentration of 0.2% and incubated at 37°C for 20 minutes. The resulting product DNA was analyzed by 1% (w/v) agarose gel electrophoresis.

Mouse urinary tract infection

Cells of strains UPEC16-WT and UPEC16 Δ *hipH* were grown to exponential phase with an OD600 of 0.3, respectively. The cells were harvested and washed with PBS, followed by resuspension in equal volumes of PBS. Bladder infection was conducted following previously described procedures with slight modifications (Pang et al, 2022 [DOI](#)). 8-week female BALB/c mice were anesthetized using isoflurane. 2×10⁸ CFU of each strain were transurethrally inoculated by use of 25G blunt needle. Two rounds of infection were performed with 24-hour interval. At 48-hour post-infection, the mice were sacrificed by cervical dislocation, and the bladders were aseptically removed for following IBC counting assay and persister counting assay. Animal studies were approved by the Animal Care Ethics Committee of Medical Research Institute, Wuhan University. We confirm that all experiments conform to the relevant regulatory standards.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 software for Windows. Significance was ascertained by two-tailed Student's t test or Two-way ANOVA. Error bars represent the standard deviations of the mean from at least three independent experiments. A value of P<0.05 was considered significant. “*” indicate significant differences (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).

Data availability

This study includes no data deposited in external repositories.

Acknowledgements

We thank Profs. Hongbin Shu (Wuhan University) and Fan Bai (Peking University) for valuable discussions. We also thank the members of our laboratory for helpful discussion. This work is supported by the grants to Y.P. from the National Key R&D Program of China (2021YFC2701602), the Natural Science Foundation of China (31970089), Science Fund for Distinguished Young Scholars of Hubei Province (2022CFA077), the Fundamental Research Funds for the Central Universities (2042022dx0003). This work is also supported by the grants to Y.Z. for the National Science Fund for Distinguished Young Scholars (82025011) and C.G. from the Natural Science Foundation of Yunnan Province of China (202001BB050005). We also thank all the staff in the Core Facilities of Medical Research Institute at Wuhan University and the Core Facilities at School of Life Sciences at Peking University for their technical support.

Conflict of interest

Authors declare that they have no competing interests.

Tables and their legends

Expanded View Figure legends

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-His-tag-mAb	MBL	Cat#D291-3; RRID: AB_10597733
HRP-Goat Anti-Mouse IgG (H+L)	ABclonal	Cat#AS003; RRID: AB_2769851
Strains and plasmids		
<i>Escherichia coli</i> strain MG1655	Yale Genetic Stock Center	CGSC#6300
<i>Escherichia coli</i> strain BW25113	Yale Genetic Stock Center	CGSC#7636
BW25113 KEIO library	Dharmacon (GE life sciences)	Cat#OEC4988
UroPathogenic <i>Escherichia coli</i> UPEC16	First Affiliated Hospital of Kunming Medical University	N/A
UPEC16 Δ <i>hipH</i>	This paper	N/A
UPEC16 Δ <i>ara</i>	This paper	N/A
UPEC16 p15A::c-di-GMP-sensor	This paper	N/A
UPEC16 Δ <i>ara</i> pBAD::vector	This paper	N/A
UPEC16 Δ <i>ara</i> pBAD:: <i>pdeH</i>	This paper	N/A
UPEC16 Δ <i>ara</i> pBAD:: <i>dgcZ</i>	This paper	N/A
UPEC16 p15A::c-di-GMP-sensor P- <i>hipH-bfp</i>	This paper	N/A
MG1655 p15A::c-di-GMP-sensor	This paper	N/A
MG1655 Δ <i>ara</i> pBAD::vector p15A::c-di-GMP-sensor	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>dosC</i> p15A::c-di-GMP-sensor	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>dgcZ</i> p15A::c-di-GMP-sensor	This paper	N/A
MG1655 Δ <i>ara</i> <i>hipH-bfp</i> pBAD::vector	This paper	N/A
MG1655 Δ <i>ara</i> <i>hipH-bfp</i> pBAD:: <i>dgcZ</i>	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>dgcZ</i> -m	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>hipH</i> -His (or <i>hipH</i> mutants)	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>hipH</i> -mScarlet-I	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>gfp</i> -His	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>hipB</i> -His	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>mCherry</i> -His	This paper	N/A
MG1655 Δ <i>ara</i> pBAD::Vector, p15A:: <i>gam</i> -GFP & <i>hipH</i>	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>dosC</i> , p15A:: <i>gam</i> -GFP & <i>hipH</i>	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>dgcZ</i> , p15A:: <i>gam</i> -GFP & <i>hipH</i>	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>hipH</i> pT5::vector	This paper	N/A
MG1655 Δ <i>ara</i> pBAD:: <i>hipH</i> pT5:: <i>recG</i>	This paper	N/A
BW25113 pBAD:: <i>hipH</i>	This paper	N/A
BW25113 Promoter-rhaB- <i>hipH</i> pBAD:: <i>dgcZ</i>	This paper	N/A
BW25113 Δ <i>hipH</i> pBAD:: <i>dgcZ</i>	This paper	N/A
BW25113 Δ <i>recA</i> pBAD:: <i>hipH</i>	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Cycle di-GMP	APExBIO	Cat#B7839

Table S1.

Key resources table.

2'-Biotin-16-c-di-GMP	BIOLOG	Cat#B098
HipH-GFP-His	This paper	N/A
GFP-His	This paper	N/A
Ampicillin	Sangon Biotech	Cat#A610028
Chloramphenicol	Sangon Biotech	Cat#A600118
Gentamicin	Sangon Biotech	Cat#A620217
Kanamycin	Sangon Biotech	Cat#A600286
Arabinose	Sigma	Cat#V900920
IPTG	Sangon Biotech	Cat# A600168
Anhydrotetracycline	MedChemExpress	Cat#HY-118660
qPCR Primers		
recA-QP1 5-CTGCTGATCTTCATCAACCA-3	This paper	N/A
recA-QP2 5-AACAGAGGCGTAGAATTCAG-3	This paper	N/A
sulA-QP1 5-GGGCTTATCAGTGAAGTTGTC-3	This paper	N/A
sulA-QP2 5-GGCTAATCTGCATTACTTTCGT-3	This paper	N/A
tisB-QP1 5-ATGAACCTGGTGGATATCGC-3	This paper	N/A
tisB-QP2 5-TTACTTCAGGTATTTTCAGAACAGC-3	This paper	N/A
hipH-QP1 5-GATTACTGCACAACACCCTG-3	This paper	N/A
hipH-QP2 5-GAAGAACCCACAATTTCTCCTG-3	This paper	N/A
Software and algorithms		
Fiji	Schindelin et al., 2012	https://fiji.sc/ ; RRID: SCR_002285
FlowJo	Treestar, Inc.	https://www.flowjo.com/

Table S1. (continued)

Fig. S1.

A c-di-GMP biosensor was used in UPEC16 strain.

(A) The phylogenetic analysis has revealed that UPEC16 shares close genetic relatedness with well-characterized UPEC reference strains such as CFT073 and UTI89. (B) Schematic overview of c-di-GMP sensor. The design of c-di-GMP biosensor is based on fluorescent protein mVenus^{NB}, with MrkH inserted between residues Y145 and N146. Conformational changes in MrkH, induced upon binding to c-di-GMP, result in reduced fluorescence of the c-di-GMP sensor. A red fluorescent protein mScarlet-I is co-expressed under the regulation of the *proC* promoter. (C-E) Frequency distribution of c-di-GMP levels (R^{-1} score) within the planktonic subpopulation from the static biofilm system harboring c-di-GMP biosensor at 0.5 h (C), 2 h (D), 3.5 h (E).

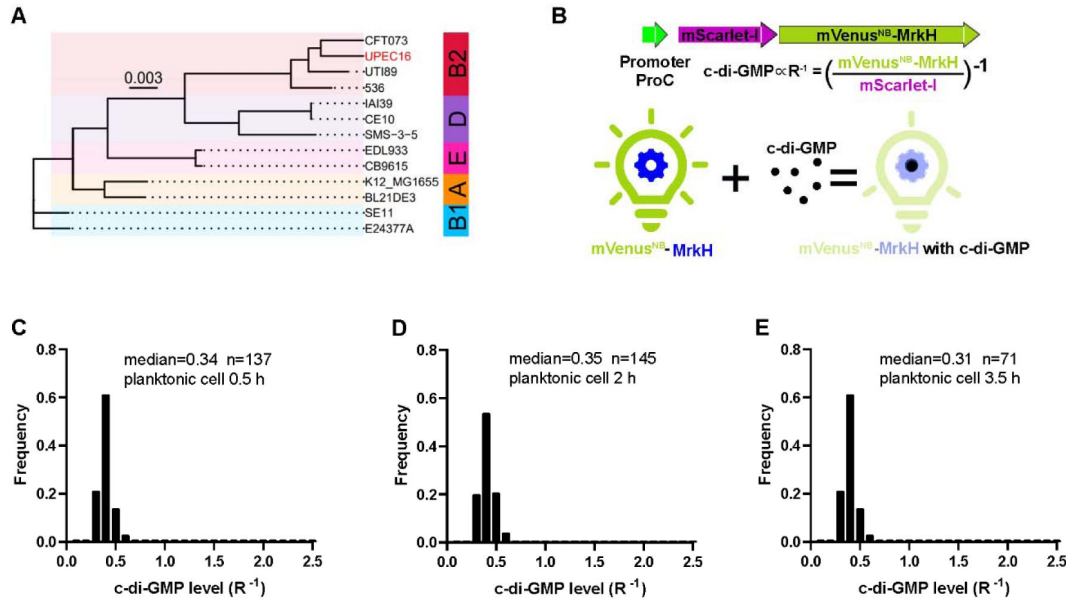
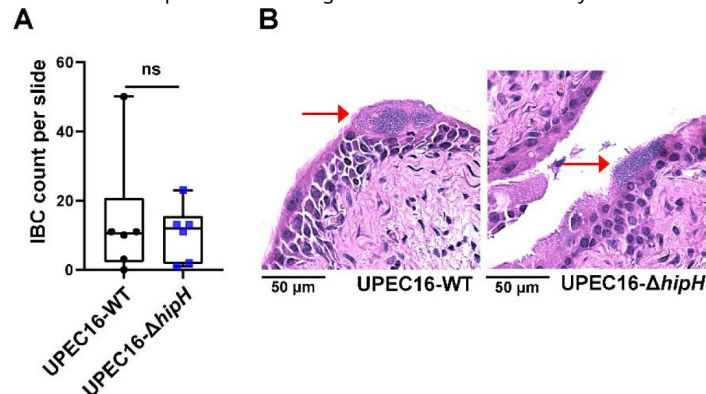


Fig. S2.

UPEC16 infection of mouse bladder.

(A) Quantification of intracellular bacterial communities (IBC) count per slide within the epithelial cells lining the bladder lumen infected by UPEC16-WT or UPEC16- $\Delta hipH$. (n=6). (B) Histological examinations of H&E stained UPEC16-WT or UPEC16- $\Delta hipH$ infected bladder tissue sections. Representative images of IBC were indicated by red arrows.



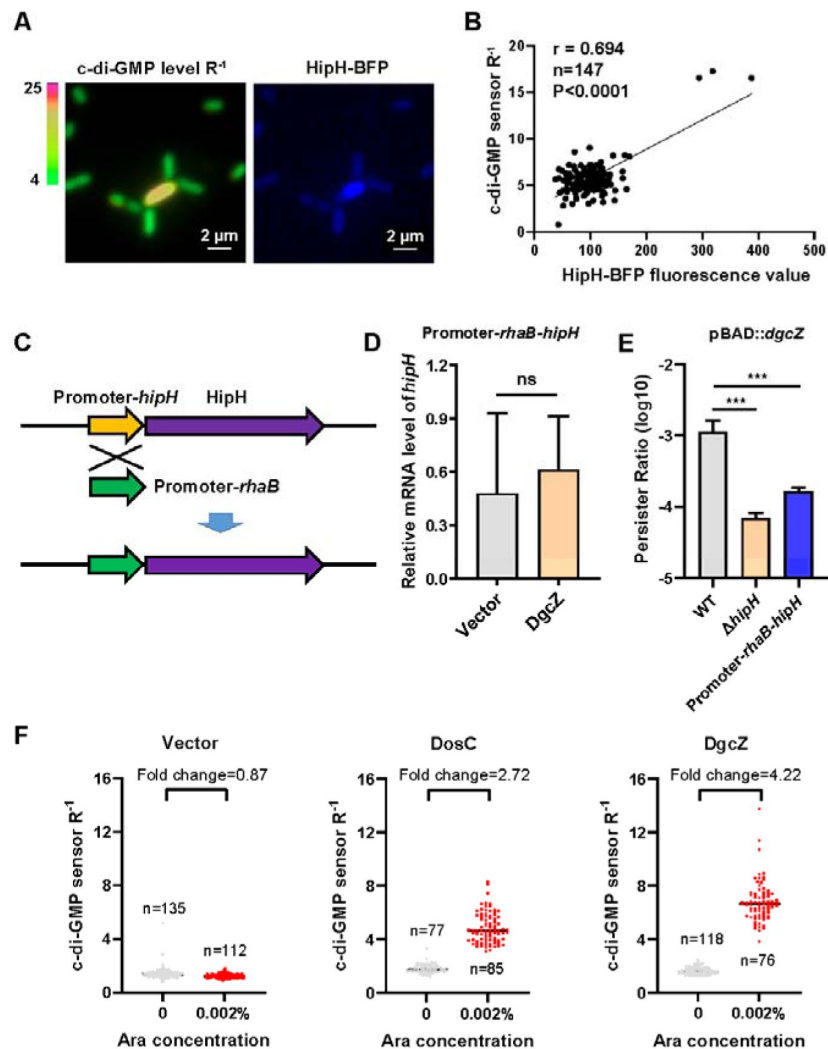


Fig. S3.

The correlation between the levels of c-di-GMP and HipH.

(A) Representative fluorescent images showing HipH-BFP fluorescence and c-di-GMP level R^{-1} . UPEC16 strain with the c-di-GMP sensing system and BFP-labeled HipH were cultured statically five-hours in microtiter dishes. (B) Correlation analysis between HipH-BFP fluorescence values and c-di-GMP level R^{-1} scores from (A). (C) Schematic representation of the promoter modification of the *hipH* gene. (D) Relative expression level of *hipH* in BW25113 Promoter-*rhaB*-*hipH* strain under varying levels of cellular c-di-GMP, determined by RT-qPCR and normalized to the levels of 16S rRNA. DgcZ was induced by 0.002% arabinose for 2 hours using the pBAD plasmid ($n=3$). (E) Determination of persister ratios in BW25113 strains overexpressing DgcZ with 0.002% arabinose induction for 2 hours using the pBAD plasmid. Persister counting was performed following antibiotic treatment (ampicillin, 150 μ g/ml, 3 hours at 37°C with shaking at 220 rpm) ($n=3$). (F) Determination of relative cellular concentrations of c-di-GMP by c-di-GMP sensing system in MG1655 cells overexpressing DosC or DgcZ, with 0.002% arabinose induction for 2 h. Error bars represent standard deviations of biological replicates. Significance was ascertained by two-tailed Student's t test. Statistical significance is denoted as ****, $P<0.0001$.

Fig. S4.

HipH induced genome instability.

(A) The cells length was detected in MG1655 strains which overexpressed HipH induced by 0.002% arabinose for 2 h under a light microscope. (B) The relative mRNA level of some SOS response genes, *recA*, *sulA* and *tisB*, were detected by qPCR in MG1655 strains which overexpressed HipH or HipH-ΔHTH induced by 0.002% arabinose for 3 h. The mRNA level was normalized by 16S rRNA level (n=3). (C) The ratio of cells with growth potential in different strains, WT: overexpressing HipH in BW25113 WT. Δ*recA*: overexpressing HipH in BW25113 Δ*recA* strain (n=3). HipH was induced by 0.002% arabinose for 2 h. (D) The ratio of cells with growth potential were detected in MG1655 strains which overexpressed HipH induced by 0.002% arabinose and overexpressed RecG induced by 1 mM IPTG for 2 h (n=3). Error bars represent standard deviations of biological replicates. Significance was ascertained by two-tailed Student's t test (A-B) or Two-way ANOVA (C-D). Statistical significance is denoted as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

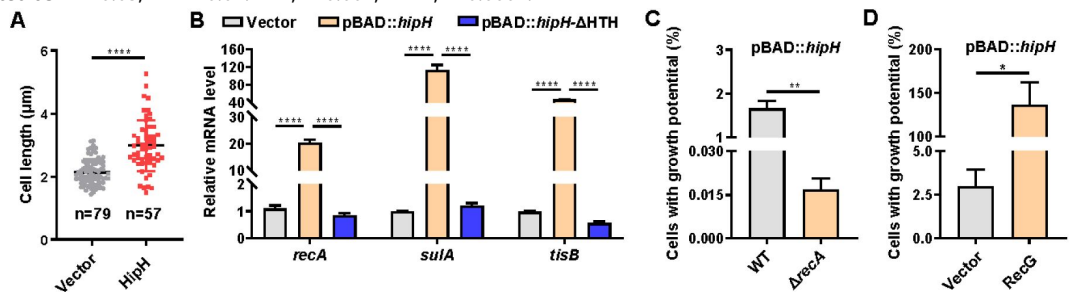
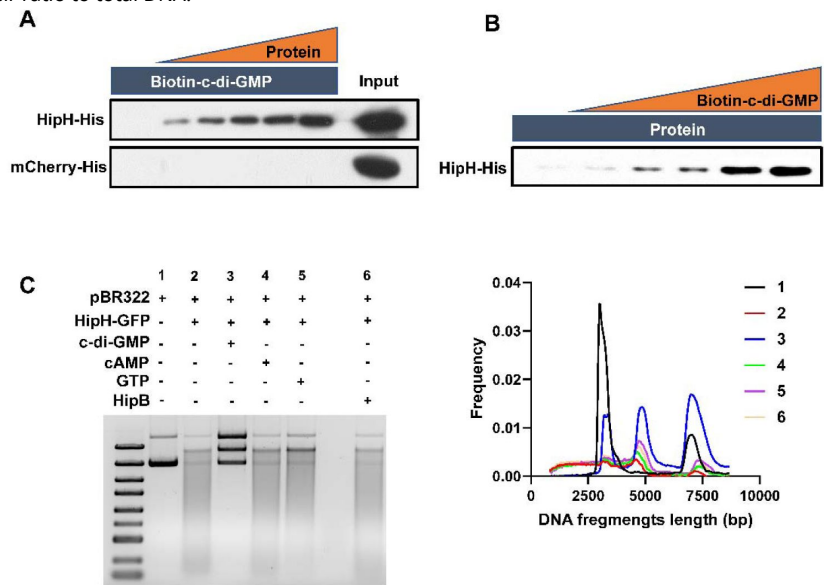


Fig. S5.

c-di-GMP binding inhibits HipH DNA Cleavage Activity.

(A) Lysates from cells overexpressing HipH-His or mCherry-His was added in gradient concentrations (0, 15, 30, 60, 120, 240 μg), with biotin-c-di-GMP was added at a constant concentration (8 μM). (B) Lysates from cells overexpressing HipH-His was added at a constant concentration (240 μg), and biotin-c-di-GMP was added in gradient concentrations (0, 0.5, 1, 2, 4, 8 μM). (C) Left panel: In vitro DNA cleavage assay using 500 ng of supercoiled plasmid DNA. A concentration of 200 nM HipH-GFP-His was employed in the cleavage reaction at 37°C for 1 hour. Subsequently, 5 mM c-di-GMP, 2.5 mM cAMP, 5 mM GTP, or 200 nM HipB-His were added to the cleavage reaction. Right panel: Illustration of the relationship between the length of DNA fragments and their ratio to total DNA.



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Editors

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University of Geneva, Geneva, Switzerland

Reviewer #2 (Public Review):

Summary:

Hebin et al reported a fascinating story about antibiotic persistence in the biofilms. First, they set up a model to identify the increased persisters in the biofilm status. They found that the adhesion of bacteria to the surface leads to increased c-di-GMP levels, which might lead to the formation of persisters. To figure out the molecular mechanism, they screened the E.coli Keio Knockout Collection and identified the HipH. Finally, the authors used a lot of data to prove that c-di-GMP not only controls HipH over-expression but also inhibits HipH activity, though the inhibition might be weak.

Strengths:

They used a lot of state-of-the-art technologies, such as single-cell technologies as well as classical genetic and biochemistry approaches to prove the concept, which makes the conclusions very solid. Overall, it is a very interesting and solid story that might attract diverse readers working with c-di-GMP, persisters, and biofilm.

Comments on the revised version:

All my concerns have been addressed.

<https://doi.org/10.7554/eLife.99194.2.sa1>

Author response:

The following is the authors' response to the original reviews.

eLife assessment

This preprint explores the involvement of cyclic di-GMP in genome stability and antibiotic persistence regulation in bacterial biofilms. The authors proposed a novel mechanism that, due to bacterial adhesion, increases c-di-GMP levels and influences persister formation through interaction with HipH. While the work may provide useful insights that could attract researchers in biofilm studies and persistence mechanisms, the main findings are inadequately supported and require further validation and refinement in experimental design.

We sincerely thank eLife for the thorough assessment of our manuscript. We appreciate the constructive criticism and see it as an opportunity to strengthen our research. In response to the reviewers' comments and suggestions, we have made significant improvements to our study. We have refined our experimental design and conducted additional experiments to provide more robust evidence supporting our findings. We believe that with these additional experiments and refinements, our study provides robust evidence for this novel mechanism, contributing significantly to the fields of biofilm research and bacterial persistence.

Public Reviews:**Reviewer #1 (Public Review):**

The authors propose a UPEC TA system in which a metabolite, c-di-GMP, acts as the AT with the toxin HipH. The idea is novel, but several key ideas are missing in regard to the relevant literature, and the experimental design is flawed. Moreover, they are absolutely not studying persister cells as Figure 1b clearly shows they are merely studying dying cells since no plateau in killing (or anything close to a plateau) was reached. So in no way

has persistence been linked to c-di-GMP. Moreover, I do not think the authors have shown how the c-di-GMP sensor works. Also, there is no evidence that c-di-GMP is an antitoxin as no binding to HipH has been shown. So at best, this is an indirect effect, not a new toxin/antitoxin system as for all 7 TAs, a direct link to the toxin has been demonstrated for antitoxins.

Thank you for your constructive comments on our manuscript. Your insights have prompted us to revisit our data and experimental design, leading to significant improvements in our study.

(1) Clarification on Persister Cell Detection: We sincerely appreciate your astute observation regarding the interpretation of our killing curve in Figure 1B. Upon careful re-examination, we concur that our initial methodology had limitations in revealing the characteristic biphasic pattern associated with persister cells. To address these limitations, we have implemented two key modifications: shortening the sampling interval and extending the antibiotic treatment duration. These adjustments have resulted in an updated killing curve that now exhibits a more pronounced biphasic pattern and a prominent plateau in the late stage of killing, as illustrated in Response Figure 1. This refined pattern aligns with established characteristics of persister cell behavior in antibiotic tolerance studies, providing a more accurate representation of the persister population dynamics in our experimental system. We believe these methodological enhancements significantly improve the reliability and interpretability of our results, offering a clearer insight into the persister cell phenomenon under investigation.

(2) Validation of c-di-GMP Sensor: We appreciate your point about the c-di-GMP sensor. The c-di-GMP sensor, developed by Howard C. Berg's team, is specifically designed to detect relative intracellular concentrations of c-di-GMP in *Escherichia coli* cells. This capability is crucial for understanding the dynamic regulation of c-di-GMP during bacterial responses to environmental stimuli. We have expanded our explanation of the sensor's detection mechanism in lines 138-146 of the manuscript, detailing how it functions to reflect changes in c-di-GMP levels within the cells accurately. The mechanism operates through a series of signaling events that are initiated when c-di-GMP binds to the sensor, leading to measurable outputs that correlate with intracellular concentrations. Additionally, we have provided a schematic chart in Figure S1B to visually support our description regarding the sensor. This figure demonstrates the sensor's responsiveness and specificity in detecting fluctuations in c-di-GMP levels, effectively linking the signaling molecule to cellular behavior. We hope these additions clarify the role of the c-di-GMP sensor in our research and address your concerns regarding its functionality.

(3) HipH and c-di-GMP Interaction: Our pull-down experiments presented in Figure 5A-E provide robust and compelling evidence for a direct physical interaction between HipH and c-di-GMP, and the effects of their interaction reminiscent of toxin-antitoxin systems. Yet we acknowledge c-di-GMP is not a traditional antitoxin since it is not genetically linked to HipH. We have revised our terminology to "TA-like system" to reflect this difference more accurately.

Weaknesses:

(1) L 53: biofilm persisters are no different than any other persisters (there is no credible evidence of any different persister cells) so this reviewer suggests changing 'biofilm persisters' to 'persisters' throughout the text.

Thank you for your thoughtful consideration. Upon careful consideration of the current scientific literature, we agree that there is no substantial evidence supporting a distinct

category of persister cells specific to biofilms. We have systematically replaced 'biofilm persisters' with 'persisters' throughout the manuscript.

(2) L 51: persister cells do not mutate and, once resuscitated, mutate like any other growing cell so this sentence should be deleted as it promotes an unnecessary myth about persistence.

We sincerely appreciate your astute observation regarding the inaccuracy in line 51. We have removed the sentence in question from line 51. And we also have thoroughly reviewed the entire manuscript to ensure no similar misconceptions are present elsewhere in the text.

(3) L 69: please include the only metabolic model for persister cell formation and resuscitation here based on single cells (e.g., doi.org/10.1016/j.bbrc.2020.01.102, <https://doi.org/10.1016/j.isci.2019.100792>); otherwise, you write as if there are no molecular mechanisms for persistence/resuscitation.

Thank you for your valuable suggestion. We appreciate the opportunity to enhance the scientific context of our manuscript. We have added a brief explanation of how ppGpp mediates ribosome dimerization, leading to persistence, and how its degradation triggers resuscitation [1-3] (lines 68-74). We have described the role of cAMP-CRP in regulating persistence through its effects on metabolism and stress responses [4, 5] (lines 74-78). We also explore potential interactions or synergies between our proposed mechanisms and these established metabolic models [6] (lines 383-409). We believe this revision significantly enhances our manuscript by providing a more accurate representation of the current state of knowledge in the field and demonstrating how our work builds upon and contributes to existing models of bacterial persistence.

(4) The authors should cite in the Intro or Discussion that others have proposed similar novel TAs including a ppGpp metabolic toxin paired with an enzymatic antitoxin SpoT that hydrolyzes the toxin (<http://dx.doi.org/10.1016/j.molcel.2013.04.002>).

We are grateful for your expertise in pointing out this crucial reference. We sincerely appreciate your suggestion to include the reference to previously proposed novel toxin-antitoxin (TA) systems, particularly the ppGpp-SpoT system [6]. In light of this reference, we have expanded our discussion to include: 1) A brief overview of the ppGpp-SpoT system as a novel TA-like mechanism. 2) Comparisons between the ppGpp-SpoT system and our findings on the HipH-c-di-GMP interaction. 3) Reflections on how these systems challenge and expand traditional definitions of TA systems (lines 383-409). We believe this addition significantly enhances the context and strengthens the rationale for considering the HipH-c-di-GMP interaction as a TA-like system. Thank you for your valuable input in helping us situate our research within the broader landscape of TA system biology.

(5) Figure 1b: there are no results in this paper related to persister cells. Figure 1b simply shows dying cells were enumerated. Hence, the population of stressed cells increased, not 'persister cells' (Figure 1f), in the course of these experiments.

We sincerely appreciate your astute observation regarding the interpretation of our killing curve in Figure 1B. Upon careful re-examination, we concur that our initial methodology had limitations in revealing the characteristic biphasic pattern associated with persister cells. To address these limitations, we have implemented 1) Shortened sampling interval: We have reduced the interval between measurements to one hour. 2) Extended sampling duration: The total duration of sampling has been increased to 6 hours (Response Figure 1). The updated killing curve now exhibits a more pronounced biphasic pattern and a prominent plateau in the late stage of killing: 1) Initial rapid decline: From 0-1hours, we observe a steep

decrease in bacterial survival (slope $\approx -3 \sim -1.8$); 2) Slower decline phase: From 4.5-6 hours, the rate of decline is markedly reduced (slope $\approx -0.17 \sim -0.06$). This pattern aligns more closely with established characteristics of persister cell behavior in antibiotic tolerance studies.

(6) Figure S1: I see no evidence that the authors have shown this c-di-GMP detects different c-di-GMP levels since there appears to be no data related to varying c-di-GMP concentrations with a consistent decrease. Instead, there is a maximum. What are the concentration of c-di-GMP on the X-axis for panels C, D, and E? How were c-di-GMP levels varied such that you know the c-di-GMP concentration?

We appreciate your point about the c-di-GMP sensor. To address this, we have included additional data on the sensor's mechanism and validation. The sensor, developed by Howard C. Berg's team, is designed for detecting intracellular c-di-GMP concentrations in *E. coli* [7].

Sensor Design and Mechanism : The sensor developed for detecting c-di-GMP levels in *Escherichia coli* cells is based on a single fluorescent protein biosensor. The protein includes a Fluorescent Protein Base and a c-di-GMP Binding Domain. The fluorescent protein base is mVenusNB, which is the fastest-folding yellow fluorescent protein (YFP). The c-di-GMP binding domain is the MrkH protein is inserted between Y145 and N146 of mVenusNB. MrkH is a transcription factor with a high affinity for c-di-GMP. When MrkH binds to c-di-GMP, it undergoes a significant conformational change. The amino-terminal domain of MrkH rotates 138° relative to its carboxyl-terminal domain upon c-di-GMP binding. This rotation disrupts the mVenusNB chromophore environment, resulting in reduced fluorescence. The sensor system co-expresses mScarletI, a bright, rapidly folding red fluorescent protein. mScarletI serves as a reference for ratiometric measurements. Such design allows for ratiometric measurement of real-time monitoring of c-di-GMP levels in individual cells and control of variations in protein expression levels between cells. This enables the observation of dynamic changes in c-di-GMP concentration, such as the increase seen after *E. coli* surface attachment.

Functioning and Accuracy: The sensor is designed to detect c-di-GMP in the 100 to 700 nM range, which is the physiological range in *E. coli*. The use of a low copy plasmid for expression ensures detection at low concentrations. The ratio (R) of mVenusNB to mScarletI fluorescence emission is measured for individual cells. The sensor shows at least a twofold dynamic range between low and high c-di-GMP conditions. Cells with low c-di-GMP (expressing phosphodiesterase PdeH) show higher R values compared to cells with high c-di-GMP (expressing constitutively active diguanylate cyclase WspR:D70E). A mutant biosensor (Sensor*) with the R113A mutation in MrkH is used as a control. This mutation eliminates c-di-GMP binding ability, allowing differentiation between specific c-di-GMP effects and other cellular changes.

This biosensor system provides a sophisticated tool for visualizing and quantifying c-di-GMP levels in individual bacterial cells with high sensitivity and temporal resolution. By combining a c-di-GMP-sensitive fluorescent protein with a reference fluorescent protein and utilizing ratiometric analysis, the system can accurately reflect changes in intracellular c-di-GMP levels while controlling for other cellular variables.

We have expanded our explanation of its detection mechanism in lines 138-146 and Figure S1B.

(7) The viable portion of the VBNC population are persister cells so there is no reason to use VBNC as a separate term. Please see the reported errors often made with nucleic acid staining dyes in regard to VBNCs.

We appreciate the opportunity to clarify the distinction between VBNC cells and persister cells in our manuscript. It is essential to recognize that VBNC cells and persister cells represent two fundamentally different states of bacterial dormancy. While both may exhibit viability under certain conditions, persister cells are characterized by their ability to resuscitate and grow when environmental conditions become favorable [8]. In contrast, VBNC cells are in a deep dormant state where they cannot be revived through normal culture conditions [9, 10]. This distinction is critical for accurately representing bacterial survival strategies and population dynamics, which is why we maintain the use of the term VBNC separately from persister cells. We have added related references in lines 259.

Regarding the reported errors associated with nucleic acid staining dyes for identifying VBNC cells, we acknowledge that these methods can exhibit limitations. Specifically, nucleic acid stains may fail to reliably differentiate between metabolically active and inactive cells, leading to inaccuracies in quantifying the true VBNC population [11]. In our study, we have opted to utilize propidium iodide (PI) staining to assess cell viability more accurately, as it effectively distinguishes dead cells from viable cells based on membrane integrity [12]. By employing this methodology, we ensure a more precise estimation of the VBNC proportion without conflating it with persister cell dynamics.

Reviewer #2 (Public Review):

Summary:

Hebin et al reported a fascinating story about antibiotic persistence in the biofilms. First, they set up a model to identify the increased persisters in the biofilm status. They found that the adhesion of bacteria to the surface leads to increased c-di-GMP levels, which might lead to the formation of persisters. To figure out the molecular mechanism, they screened the E.coli Keio Knockout Collection and identified the HipH. Finally, the authors used a lot of data to prove that c-di-GMP not only controls HipH over-expression but also inhibits HipH activity, though the inhibition might be weak.

Thank you for your insightful summary of our research. We greatly appreciate your thoughtful consideration of our work.

Strengths:

They used a lot of state-of-the-art technologies, such as single-cell technologies as well as classical genetic and biochemistry approaches to prove the concept, which makes the conclusions very solid. Overall, it is a very interesting and solid story that might attract diverse readers working with c-di-GMP, persisters, and biofilm.

Weaknesses:

(1) Is HipH the only target identified by screening the E. coli Keio Knockout Collection?

We appreciate your inquiry about our screening process and the identification of HipH. We did not screen the entire *E. coli* Keio Knockout Collection. Our approach was more targeted, focusing on mutants relevant to enzyme activity regulation. We selected specific mutants based on their potential involvement in c-di-GMP-mediated regulatory pathways. This focused approach allowed us to efficiently identify candidates likely to be involved in persister formation. Among the screened mutants, HipH emerged as a significant hit. Its identification was particularly noteworthy due to its known role in persister formation and its potential as a regulatory target of c-di-GMP. We acknowledge that our targeted approach may have overlooked other potential candidates. We are considering a more comprehensive screening approach in future studies to identify additional targets.

(2) Since the story is complicated, a diagrammatic picture might be needed to illustrate the whole story. And the title does not accurately summarize the novelty of this study.

Thank you for your valuable feedback. We fully agree with your assessment that a visual representation would greatly enhance the clarity of our complex findings. In response to your suggestion, we have added Response Figure 2 (Fig. 6 in revised manuscript, lines 976-981) to our manuscript. This new figure provides a comprehensive visual summary of the key processes and mechanisms uncovered in our study. This graphic summary provides a clear overview of the interconnected nature of surface adhesion, c-di-GMP signaling, and HipH regulation. It also highlights the complex role of c-di-GMP in persister formation and offers readers a visual aid to better understand the molecular mechanisms underlying our findings.

We sincerely appreciate your thoughtful comment regarding the title and its reflection of the study's novelty. After careful consideration, we believe that our original title adequately captures the essence and significance of our research. We have strived to ensure that it accurately represents the scope and novelty of our work while maintaining clarity and conciseness. Nevertheless, we value your input and thank you for taking the time to provide this feedback, as it encourages us to critically evaluate our presentation.

(3) The ratio of mVenusNB to mScarlet-I (R) negatively correlates with the concentration of c-di-GMP. Therefore, R-1 demonstrates a positive correlation with the concentration of c-di-GMP. Is this method validated with other methods to quantify c-di-GMP, or used in other studies ?

We appreciate your point about the c-di-GMP sensor. To address this, we have included additional data on the sensor's mechanism and validation. The sensor, developed by Howard C. Berg's team, is designed for detecting intracellular c-di-GMP concentrations in *E. coli* [7].

Sensor Design and Mechanism : The sensor developed for detecting c-di-GMP levels in *Escherichia coli* cells is based on a single fluorescent protein biosensor. The protein includes a Fluorescent Protein Base and a c-di-GMP Binding Domain. The fluorescent protein base is mVenusNB, which is the fastest-folding yellow fluorescent protein (YFP). The c-di-GMP binding domain is the MrkH protein is inserted between Y145 and N146 of mVenusNB. MrkH is a transcription factor with a high affinity for c-di-GMP. When MrkH binds to c-di-GMP, it undergoes a significant conformational change. The amino-terminal domain of MrkH rotates 138° relative to its carboxyl-terminal domain upon c-di-GMP binding. This rotation disrupts the mVenusNB chromophore environment, resulting in reduced fluorescence. The sensor system co-expresses mScarletI, a bright, rapidly folding red fluorescent protein. mScarletI serves as a reference for ratiometric measurements. Such design allows for ratiometric measurement of real-time monitoring of c-di-GMP levels in individual cells and control of variations in protein expression levels between cells. This enables the observation of dynamic changes in c-di-GMP concentration, such as the increase seen after *E. coli* surface attachment.

Functioning and Accuracy: The sensor is designed to detect c-di-GMP in the 100 to 700 nM range, which is the physiological range in *E. coli*. The use of a low copy plasmid for expression ensures detection at low concentrations. The ratio (R) of mVenusNB to mScarletI fluorescence emission is measured for individual cells. The sensor shows at least a twofold dynamic range between low and high c-di-GMP conditions. Cells with low c-di-GMP (expressing phosphodiesterase PdeH) show higher R values compared to cells with high c-di-GMP (expressing constitutively active diguanylate cyclase WspR:D70). A mutant biosensor (Sensor*) with the R113A mutation in MrkH is used as a control. This mutation eliminates c-di-GMP binding ability, allowing differentiation between specific c-di-GMP effects and other cellular changes.

This biosensor system provides a sophisticated tool for visualizing and quantifying c-di-GMP levels in individual bacterial cells with high sensitivity and temporal resolution. By combining a c-di-GMP-sensitive fluorescent protein with a reference fluorescent protein and utilizing ratiometric analysis, the system can accurately reflect changes in intracellular c-di-GMP levels while controlling for other cellular variables.

We have expanded our explanation of its detection mechanism in lines 138-146 and Figure S1B.

(4) References are missing throughout the manuscript. Please add enough references for every conclusion.

We appreciate your feedback regarding the references in our manuscript. We acknowledge the importance of proper citation to support our conclusions and provide context for our work. In response to your comment, we have conducted a comprehensive review of our manuscript and have significantly enhanced our referencing throughout. We have added appropriate citations to support each key statement and conclusion presented in our study. These additional references provide a robust foundation for our findings and place our work within the broader context of the field. The complete list of all references, including the newly added ones, can be found at the end of this response letter as well as in the revised manuscript.

(5) The novelty of this study should be clearly written and compared with previous references. For example, is it the first study to report the mechanism that the adhesion of bacteria to the surface leads to increased persister formation?

We sincerely appreciate the opportunity to highlight and elaborate the novelty of our research. This study provides novel insights into the relationship between bacterial adhesion to surfaces and the subsequent increase in persister cell formation, which has not been explicitly detailed in previous literature. While existing research has established that biofilms typically harbor higher numbers of persister cells, this investigation not only corroborates that finding but also elucidates the mechanisms through which surface adhesion contributes to this phenomenon.

Past studies have predominantly focused on the general characteristics of persister cells and their role in biofilm resilience and antibiotic tolerance without specifically addressing the mechanistic link between adhesion and persister formation [13, 14]. For instance, previous work has shown that surface attachment leads to changes in metabolic activity and signaling pathways within bacterial cells, which could promote persistence, but it has not definitively established a causal relationship between adhesion and increased persister formation. Our study highlights that the elevation of cyclic di-GMP levels after surface adhesion triggers a cascade of physiological changes that significantly enhance the formation of persister cells. In particular, we report that adhesion-induced signaling pathways promote dormancy and tolerance to antibiotics, marking an important advancement from the previous understanding that treated persister cells might arise from random phenotypic variation during biofilm development. We have expanded our discussion in lines 366-381.

In summary, we believe this study stands as one of the first to clearly delineate the mechanism by which bacterial adhesion leads to increased persister formation, providing a valuable contribution to the current understanding of bacterial persistence and biofilm ecology. Thus, we can assert that our findings are not only novel but also essential for informing future research and therapeutic strategies aimed at managing bacterial infections.

(6) *in vitro* DNA cleavage assay. Why not use bacterial genomic DNA to test the cleaving of HipH on the bacterial genome?

Thank you for your feedback regarding our experimental approach. The decision of not directly using genomic DNA in our experiments was made after careful consideration. The high molecular weight of genomic DNA, which presents significant challenges in handling and analysis. The difficulty in extracting intact genomic DNA, which could potentially compromise the integrity of our results. The challenges associated with electrophoretic separation of such large DNA molecules, which could limit our ability to accurately interpret the data.

Instead, following established practices in molecular biology research and drawing from similar studies in the field [15-17], we opted to use plasmids as model DNA for our experiments. This approach offers several advantages: Plasmids are smaller and more manageable, making them easier to manipulate in laboratory conditions; They can be more readily extracted in intact form, ensuring the quality of our experimental material; Plasmid DNA is more amenable to electrophoretic separation, allowing for clearer and more precise analysis. Despite their smaller size, plasmids retain many of the key characteristics of genomic DNA that are relevant to our study. We believe this approach provides a robust and reliable model for our research while overcoming the practical limitations associated with genomic DNA. It allows us to investigate the fundamental principles we're interested in, while maintaining experimental feasibility and data integrity. We have added related references in lines 314 and 599.

(7) *C-di-GMP -HipH* is not a TA, it does not fit in the definition of the TA systems. You can say *C-di-gmp* is an antitoxin based on your study, but *C-di-gmp -HipH* is not a TA pair.

We appreciate your insightful feedback regarding the classification of the c-di-GMP-HipH interaction. We acknowledged that while our study suggests c-di-GMP may function as an antitoxin to HipH, the c-di-GMP-HipH pair does not constitute a classical TA system due to the lack of genetic linkage. We have replaced the term "TA system" with "TA-like system" when referring to the c-di-GMP-HipH interaction. This more accurately reflects the nature of their relationship while acknowledging that it differs from traditional TA systems.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) Either indent or skip a line to indicate a new paragraph; there is no need to do both.

Thank you for your feedback regarding the formatting of our manuscript. We have revised the formatting throughout the main text by using a single blank line to separate paragraphs, without indentation.

(2) L 77: need to define '*c-di-GMP*' without using another abbreviation; please write '*3,5-cyclic diguanylic acid*', etc.

Thank you for your valuable feedback regarding the proper introduction of abbreviations in our manuscript. We have revised line 86 to introduce the full name of c-di-GMP as "3,5-cyclic diguanylic acid". Following this initial introduction, we consistently use the abbreviation "c-di-GMP" throughout the rest of the manuscript.

Reviewer #2 (Recommendations For The Authors):

This is a fascinating story, but the title and the manuscript need careful revision to make it more clear. The novelty and logic are not very easy to follow.

(1) Figure 1B, "h" is missing

We sincerely thank you for your attentive review and for pointing out the missing "h" in Figure 1B. We have carefully reviewed and revised the figure legend in Figure 1B. The unit of time has been corrected to include "h" (hours) where appropriate, ensuring consistency and accuracy throughout the figure.

(2) Line 222, the *in vivo* mice model should be cited with the reference.

Thank you for the reminding. We have cited the following reference related to the mice model (line 231).

Pang Y, et al., (2022) Bladder epithelial cell phosphate transporter inhibition protects mice against uropathogenic *Escherichia coli* infection. *Cell reports* 39: 110698

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(13) Pan, X., et al., *Recent Advances in Bacterial Persistence Mechanisms*. Int J Mol Sci, 2023. **24**(18).

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