

Unraveling Direct Correlations between Membrane Nanodomain Reorganization and Antimicrobial Resistance Evolution in Bacterial Cells

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Bacterial drug resistance is a major global health emergency that requires newer approaches for its detection, especially those that are rapid and sensitive at the single cell level. One of the major limitations of existing antimicrobial resistance (AMR) screening is that it relies on culturing bacterial samples, which is time- and resource-intensive, or on detection of known mutations that impart resistance. Here we provide the first evidence for the existence of direct correlations between nanoscale dynamical reorganization in Gram-negative bacterial cell membranes with their evolution of phenotypic resistance under sublethal dosage of the last-line antibiotic Colistin. While super-resolution fluorescence microscopy in combination with fluorescence correlation spectroscopy enables probing dynamical lipid nanodomains on single *E. coli* cells undergoing AMR evolution, high-resolution atomic force microscopy provides information on nanoscale morphological changes in the same cell population. Interestingly, our study also reveals intricate correlations between nanoscale bacterial membrane organization and biochemical signaling responses that eventually drive the evolution of antimicrobial resistance. In addition, we detect signatures of cooperative lipid motion and dynamic heterogeneity as quantified through the non-Gaussian parameter, α_2 , for lipid number fluctuations in the illumination volume. Further, this parameter is also correlated with the evolution of resistance in the strains. Our study suggests a subtle feedback mechanism for the emergence of antimicrobial resistance which is initiated by membrane nanoscale organization and lipid dynamics leading to biochemical signaling that leads to membrane compositional changes. These compositional changes alter these membrane nanoscale parameters to mitigate the antibiotic mediated stress, and they increase the survival probability of the cell population, which thus becomes more resistant. Our study could thus lead to the development of a fundamentally new approach with high resolution and sensitivity that could be used to infer about antimicrobial resistance evolution, which could also be applicable to other Gram-negative strains and membrane-targeting antibiotics.

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I. INTRODUCTION

The Gram-negative bacterial outer membrane is characterized by its asymmetric structure, with phospholipids predominantly found in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet [1–3]. Colistin (polymyxin E) is an amphiphilic cyclic polypeptide with a positive charge that

exerts its antibacterial effects through the disruption of bacterial membranes, primarily through electrostatic interactions with LPS, used as a last resort for treating multi-drug-resistant bacterial infections [1,4–6]. Drug combinations are commonly used to prevent the evolution of resistance, but the presence of tolerant mutant will lead to the evolution of resistance [7,8]. Transmission electron microscopy (TEM) studies have demonstrated that treatment with Colistin induces significant changes in the morphology of Gram-negative bacteria [9]. Due to its nephrotoxic effects, sublethal concentrations of Colistin are generally used [10]. In the context of resistance to this antibiotic at sublethal concentrations, bacteria modify their LPS structure by adding extra carbohydrate chains [11]. When this occurs, the negative charge of the LPS molecules decreases, reducing binding affinity to Colistin [12,13], while the long-term exposure facilitates the emergence of genetically resistant mutants [14,15]. What is less known are the

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specific microscopic membrane signatures that emerge due to interaction with Colistin at sublethal short-term exposure. In addition, these membrane changes can, in turn, activate signaling pathways that elicit the emergence of resistance through modification of LPS. Given that a live Gram-negative bacterial membrane is highly complex and dynamic, studying such interactions requires simultaneous probing of the membrane with high spatial and temporal resolution. Super-resolution stimulated emission depletion (STED) microscopy combined with fluorescence correlation spectroscopy (FCS) is one such technique that has been used by us [16–18] and others [19,20] to study model membranes. In this regard, our recent studies on *E. coli* model and cell membranes using FCS revealed subtle changes in membrane dynamics due to interaction with small molecules and various antibiotics, including Colistin [21,22]. We extend our initial studies by employing higher spatial resolution probes to investigate *E. coli* outer membranes in addition to biochemical assays conducted under systematic Colistin dosage to explore the emergence of resistance and potential reversal mechanisms.

II. RESULTS

A. Growth kinetics of *E. coli* with Colistin: Acquisition and reversal of Colistin resistance

The *E. coli* 10536 strain, used here, has a minimum inhibitory concentration (MIC) of 2 μg per mL to Colistin. MIC measurements were measured using the broth microdilution (BMD) assay [details in the Methods section and illustrated in Fig. S1 of the Supplemental Material (SM) [23]]. Serial passaging experiments, as described in the Methods section and illustrated in Fig. 1, showed an eightfold higher MIC than the parental strain when incubated with 0.5 $\mu\text{g}/\text{mL}$ followed by 1 $\mu\text{g}/\text{mL}$ Colistin [Fig. 1(c)]. Interestingly, the MIC remained unchanged when the strain was further incubated with 2 or 4 $\mu\text{g}/\text{mL}$. Notably, there was no change in MIC in the presence of 0.5 μg per mL concentration. From Fig. 1(c), a noticeable shift in the MIC was evident at/equal to or above the concentration of 1 $\mu\text{g}/\text{mL}$ of Colistin. For clarity, we designate the treatment on strains as C_X , where X denotes the concentration at which the bacteria were incubated. This designation will be used from hereon. We selected the C_1 strain to investigate the nature of resistance and probe potential reversal. For the C_{1R} , the MIC reduced from 16 μg per mL to 8 μg per mL [Fig. 1(c)], suggesting that the observed resistance was phenotypic, and removal of antibiotic stress potentially led to a reversal, possibly due to dilution or degradative loss of resistance factor. To elucidate the impact of Colistin at the molecular level, quantitative gene expression analysis was performed (by qRT-PCR) to assess the transcriptional changes associated with Colistin resistance [Figs. 1(d) and 1(e)]. The genes that were specifically examined included two-component systems activated by Colistin, *pmrAB*, and *phoPQ*, genes that regulate LPS modifications, such as *mgrB*, *plsB*, and *plsC*, involved in phospholipid and lipid A biosynthesis and affect membrane integrity; *lpxA*, *lpxC*, and *lpxD*; and *acrB*, an efflux pump, which is part of the AcrAB-TolC multidrug efflux system in *E. coli*. Baseline expression levels were from *E. coli* cultured without

Colistin. Expression levels in untreated cells and reversed cultures (described above) were generally similar for most genes (baseline expression). However, upregulation of signaling systems *pmrAB*, *phoP* [Fig. 1(d)], and other downstream genes that modify lipid A *acrB*, *plsB*, *plsC*, and *lpxA* [Fig. 1(e)], was recorded in cells exposed to 1 $\mu\text{g}/\text{mL}$ Colistin. Interestingly, for almost all the genes, the expression was down-regulated (from 1 $\mu\text{g}/\text{mL}$ treated condition) in reversal setup without antibiotic, indicating that enhanced expression of these genes imparts Colistin resistance. The findings also demonstrate that Colistin-induced membrane perturbations can activate signaling if the dose is sublethal by activating both the PhoPQ and PmrAB system, followed by downstream genes. However, at the MIC, up-regulation of only *pmrAB* is noticed but not for PhoPQ, highlighting that this two-component signal transduction (TCS) mediated resistance at a sublethal dose of Colistin. It is also possible that at MIC, there is induction of these genes at early stages but the induction is lost when membrane integrity is severely compromised. To validate that the membrane integrity was indeed compromised and reversible, we performed the classical EtBr uptake experiment [24–26]. As evident in Fig. 1(f), the bacteria that were cultured in progressively higher concentrations of Colistin (C_1 , C_2) showed reduced EtBr membrane permeability, and the permeability increased again when the bacteria was cultured without Colistin (C_{1R}). To test the permeability, the different strains were exposed to a constant dosage of 4 $\mu\text{g}/\text{mL}$ Colistin in the assay. These observations set the stage for probing microscopic membrane signatures, through which we can distinguish lethal versus sublethal and transient membrane perturbations, which induce differential gene expression changes associated with Colistin resistance.

B. Membrane morphology evolution using atomic force microscopy

Given that Colistin targets Gram-negative bacterial membranes, it is natural to investigate its effects on membrane function and integrity. Since the biochemical assays were performed on a population of cells, for statistically robust comparison of these results with atomic force microscopy (AFM) it is necessary to obtain images over multiple cells. As shown in Fig. S2 of the SM [23], it is relatively easier to obtain such images with better clarity in air mode compared to liquid mode and over large numbers of cells [27–29], although the substrates used in the two cases were different. It turns out that the choice of substrate and buffer is very important in obtaining substrate-bound cells providing stable imaging over time. Further, for high-resolution imaging, air mode AFM images have been shown to have much better clarity compared to liquid mode imaging [30,31]. Hence we decided to proceed with air mode AFM imaging on poly-L-lysine (PLL) substrate.

Subpopulations of bacteria, imaged in a dried state using AFM, with smooth or rough surfaces were observed for strains C_1 and $C_{0.5}$ strains [Fig. 2(a)]. Interestingly, C_{1R} and C_2 have a large number of bacterial populations with only smooth membranes. Figure 2(b) quantifies the topography through root mean square (RMS) roughness (R_q). C_1 shows a bimodal distribution of R_q . The comparison was made with respect

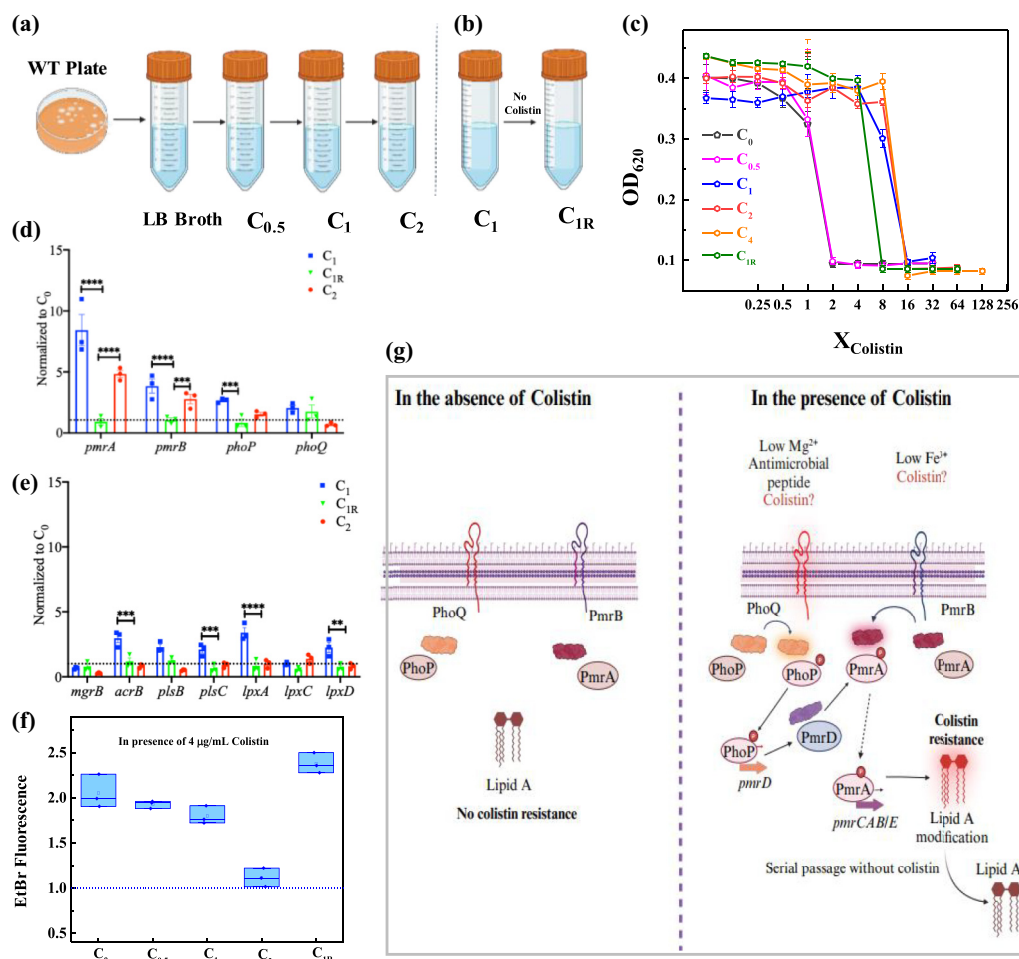


FIG. 1. (a) Serial passage protocol: *E. coli* strain 10536 cells were serially passaged every 12 h into an LB medium, with increasing Colistin concentration as indicated. (b) Resistance reversal: A sample of cells treated with $2 \mu\text{g/mL}$ colistin was cultured in LB medium without Colistin to assess the potential reversal of acquired resistance. (c) MIC of Colistin on *E. coli* was determined by measuring the absorbance at OD_{620} , serving as an indicator of bacterial cell growth. Different concentrations of Colistin ($X_{Colistin}$ in $\mu\text{g/mL}$) were tested to determine the lowest concentration at which bacterial growth was inhibited. To determine the MIC of Colistin, 1% primary inoculum of *E. coli* was seeded in the growth medium (containing increasing concentrations of Colistin) in the 96-well plate and then incubated for 20 h at 37°C . The experiments were performed in triplicate and repeated as independent biological experiments. The plot clearly shows that both the C_0 and $C_{0.5}$ cultures exhibit the MIC at $2 \mu\text{g}$ per mL of Colistin. However, a significant rise is observed at concentrations $\geq 1 \mu\text{g}$ per mL, reaching $16 \mu\text{g}$ per mL of Colistin, following which it reverts to $8 \mu\text{g}$ per mL after 12–24 serial passages without Colistin. (d),(e) Expression analysis of genes ($pmrA$, $pmrB$, $phoP$, $phoQ$, $mgrB$, $acrB$, $plsB$, $plsC$, $lpxA$, $lpxC$, $lpxD$, described in the text) involved in Colistin resistance in various conditions as indicated by qRT-PCR as described in the Methods section. The fold change is the average of three independent experiments, with error bars representing the standard error of the mean. Expression levels of genes were normalized to untreated *E. coli* cells, set as a reference at 1. Statistical analysis was performed using ordinary two-way ANOVA. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; and * $P < 0.05$. (f) EtBr assay: The Y axis is the fluorescence recorded in the strains, normalized with respect to the fluorescence in the C_0 strain in the absence of any Colistin. (g) Schematic showing that the two-component system in *E. coli* starts activating while sensing Colistin in the outer membrane and responds by synthesizing modified LPS (charge-neutralized) to be transported to the OM, which the positively charged Colistin cannot disrupt.

to the topographically smooth surface of C_0 [Figs. 2(a) and 2(b)]. For the C_2 strain, the membrane again showed a smooth surface with a unimodal R_q distribution [Figs. 2(a) and 2(b)]. Notably, upon removal of Colistin pressure, the C_{1R} strain exhibited a uniformly smooth morphology across the entire population [Figs. 2(a) and 2(b)]. From the AFM results, it can be seen that the incubation of bacterial cells with Colistin initially caused surface roughness consistent with our earlier observations [22] of increased roughness, while there was very little roughness increase for a highly genetically modified strain. However, when the cells were serially incubated at a

higher concentration of Colistin, or if the Colistin pressure was removed, the membrane property resembled that of the primitive C_0 .

To obtain further insights into possible correlations between nanoscale morphology variations and AMR evolution, we used higher resolution AFM. Recent reports [32,33] suggest evidence of phase separation in the bacterial outer membrane involving two major components—LPS and outer membrane porins (OMPs). Figure 2(c) shows the high-resolution AFM phase map of the C_0 outer membrane (top panel) indicating the presence of nanodomains. However,

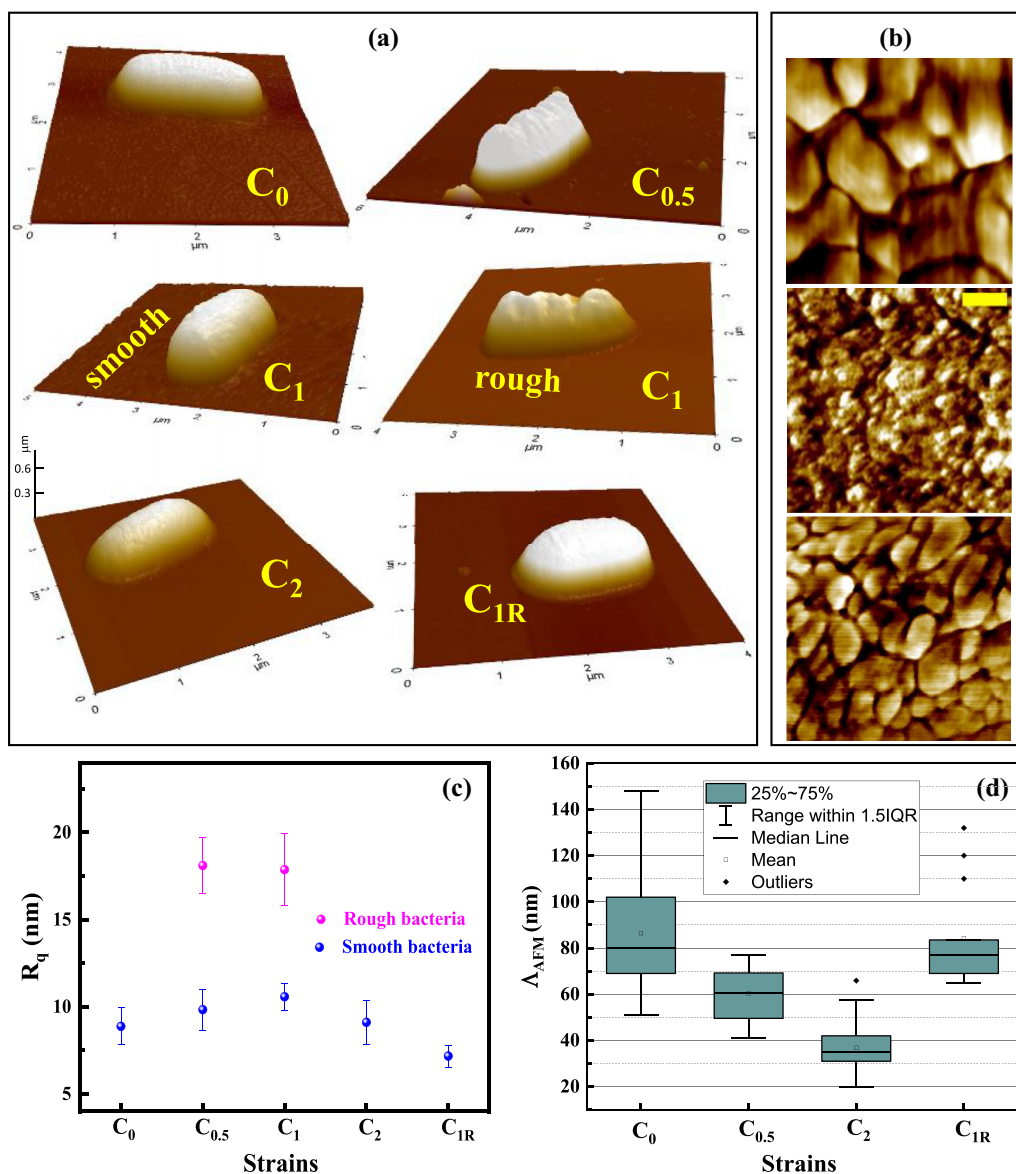


FIG. 2. (a) AFM topographical maps of C_0 , $C_{0.5}$, C_1 , C_2 , and C_{1R} *E. coli* strains. The height scale is consistent across all 3D images; for clarity, the z -axis label is shown in only one of the images. Images show C_1 outer membranes having either smooth or rough morphology. (b) Plot of surface roughness of bacteria for the various strains of *E. coli*. $C_{0.5}$ and C_1 strains show two subpopulations showing either smooth or rough outer membrane. The surface roughness [root mean squared (RMS) values, R_q] of the bacteria was averaged over approximately 10 different bacterial surfaces, which provided the statistical deviation from the mean in the roughness values. Part (c) shows the high-resolution phase maps of C_0 , C_2 , and C_{1R} *E. coli* strains, collected from a portion of the entire membrane. Scale bar for the phase maps = 100 nm. The maps show apparent nanodomain structures of different sizes. Part (d) shows the plot of average nanodomain size (Λ_{AFM}) of C_0 , C_2 , and C_{1R} strains. The box in the box plot bounds the 25th percentile to the 75th percentile of the Λ_{AFM} distribution. The error bar shows the Interquartile region encompassing the 5th percentile to the 95th percentile (scatter plot shown in Fig. S5 of the SM [23]).

given that air mode imaging could likely introduce drying-induced artifacts for the live cells [29,34], we acquired time-dependent AFM images upon drying and did not observe any significant changes in the nanodomain morphology (see Fig. S3 of the SM [23]). The height of these patches in C_0 membrane [Fig. 2(c) (top panel)] compared to the darker background is a few nm (Fig. S4 of the SM [23]) [32]. The phase contrast in the phase image suggests that the patches are contained within a boundary. This suggests the possibility of porins being present on the periphery of these nanodomains. Interestingly, for the C_2 strain of *E. coli*, similar patches were

present but with significantly smaller sizes [Fig. 2(c) (middle panel)]. Interestingly, for the C_{1R} strains the size of the patches became larger again. To quantify these variations further, we defined a parameter, nanodomain size Λ_{AFM} , shown in Fig. 2(d) (scatter plot in Fig. S5 of the SM [23]). The decrease in Λ_{AFM} observed in the C_2 strain was clearly reversed in the C_{1R} strain.

It can thus be commented that as the *E. coli* strain gains resistance to Colistin, there is a possibility of structural rearrangement of the LPS and the porin network to alter the nanodomain sizes. When the resistant strain (C_1) was allowed

to grow without Colistin, the MIC reduced, and along with it, the nanodomains regained a larger size comparable to C_0 . Thus AFM data provide a method to quantify single bacterial membrane morphology and correlate them with the parameters extracted from biochemical assays on *E. coli* cultures. Is there a similar correlation between single bacterial cell membrane nanoscale dynamical parameters and those extracted from the biochemical assays? In the next section, we discuss results from spot variation (SV) FCS measurements on the same *E. coli* strains discussed above.

C. SV-STED FCS measurements capture membrane dynamic reorganization

We now present the results of STED-FCS, which serve as a key component of this study, to investigate the mechanism underlying resistance reversal. For these experiments, bacterial cells were immobilized using PLL and imaged under liquid buffer conditions to maintain a physiologically relevant environment. This is also consistent with the imaging conditions for AFM as discussed in the earlier section, with the exception that those images were collected in air.

The average particle number in the observation spot in confocal or STED mode is denoted by $\bar{N}$ and the fluctuation in the particle number is represented by $\Delta N(t)$. The total number of particles at any given time can be written as

$$N(t) = \bar{N} + \Delta N(t). \quad (1)$$

By measuring the emission intensity $[I(t) = \langle I \rangle + \Delta I(t)]$, where $\langle I \rangle$ and $\Delta I(t)$ represent time-averaged intensity and fluctuation, respectively, it is possible to calculate the particle number fluctuations $N(t)$ of fluorescent molecules entering and leaving the focal plane of a confocal microscope observation spot during excitation. In FCS, the autocorrelation function $g_2(\tau)$ from the intensity signal $I(t)$ measured in the microscope is calculated using

$$g_2(\tau) = \frac{\langle \Delta I(t) \Delta I(t + \tau) \rangle_t}{\langle I(t) \rangle_t^2}, \quad (2)$$

where $\langle \rangle_t$ denotes that the physical quantity was averaged over the time variable t . The correlation data were normalized and fitted to single or multicomponent 2D diffusion equation as follows:

$$g_2(\tau) = G_\infty + \left[\frac{1}{N} \frac{(1 - \theta_t + \theta_t e^{-\frac{\tau}{\tau_t}})}{1 - \theta_t} \frac{\rho}{(1 + \frac{\tau}{\tau_D})^\alpha} \right], \quad (3)$$

where G_∞ is the background of the curve where the autocorrelation decays to 0. α is the anomaly parameter, which is 1 for Brownian diffusion and deviates from 1 in the case of the subdiffusive or superdiffusive nature of diffusion. θ_t is the fraction of molecules that are present in the triplet state, τ_t is the relaxation time in the triplet state, ρ is the fraction of diffusing molecules, which is unity in these fits, and τ_D is the diffusion time. We implement spot-variation FCS with the help of continuous variation of the point spread function (PSF) using STED. The obtained data for τ versus d^2 can be interpreted in terms of FCS diffusion laws of the

form [17,35–39]

$$\tau_D = \frac{d^2}{D_{\text{eff}} 8 \ln 2} + t_0. \quad (4)$$

Here d is the diameter of the confocal/STED PSF and t_0 is the y-intercept, and D_{eff} is the effective lipid diffusion coefficient.

Figure 3(a) shows variation of τ_D with d^2 for C_0 and C_2 strains. We observe two regimes of membrane lipid dynamics for the C_0 strain separated by the parameter ξ , while for the C_2 strain we observe a single regime of lipid diffusion. The diffusion coefficients observed in the C_0 strain closely resemble the outer membrane diffusivity observed in the literature [40] and from our earlier study [21]. To investigate further, we extracted spheroplasts and OMVs and labeled them with Nile red. As expected, Nile red stained both the IM and OM; however, the majority of the diffusion contribution appears to originate from the OM (Fig. S13 of the SM [23]). In Fig. 3(b), we observe the plots revealing the presence of multiple regimes of membrane lipid dynamics for all the strains based on the C_1 strain. First, the data for C_1 show clear inversion of the slopes in the two regimes compared to the data for C_0 or $C_{0.5}$ (Fig. S10A of the SM [23]) strains with the emergence of a regime with $t_0 > 0$ for $d > \xi$. This aspect is particularly interesting because we observed a sudden eightfold increment in the MIC of Colistin compared to C_0 based on the BMD assay discussed in Fig. 1(c). Since C_1 was the critical strain where the STED-FCS plot significantly altered from C_0 , we tested the effects of Colistin by directly incubating C_0 in 1 $\mu\text{g/mL}$ of Colistin and allowing it to grow to saturation stage. The sample was subcultured from the saturation stage by taking an initial secondary inoculum concentration of 0.01% of the saturated culture and allowing it to reach midlogarithmic phase. This strain was called C_{1D} . The STED-FCS plot for C_{1D} reveals the presence of three length-scale-dependent diffusive regimes. The trend partly resembled that of C_0 ($d > \xi_{1D}^H$ and $d < \xi_{1D}^L$) and partly that of C_1 (due to the middle regime having $t_0 > 0$). Finally, the data for the C_{1R} strain show inversion in terms of sign of t_0 and the slope compared to C_1 but similar to that of C_0 . To further quantify these data, we plot the ratio of D_L and D_H in Fig. 3(c) representing an estimate of mobility contrast between the different regimes of lipid similar to our recent report [18]. Interestingly, we observe that this parameter once again shows a minima around the C_1 strain and increases for C_{1R} strain similar to variations of relevant parameters in Figs. 1 and 2. Of course, for the C_2 strain, this ratio takes a trivial value of 1, suggesting spatial homogenization of membrane lipid dynamics at least within the spatial resolution of our measurements. Further, when we extracted a nanodomain size parameter, Λ_{STED} , from the respective values of t_0 , we observed a very interesting trend in Fig. 3(d). Λ shows a minimum for C_1 and is also similar for C_2 . However, the value starts rising again for C_{1R} , suggesting that the trends observed for the evolution of resistance are clearly reflected in the membrane nanoscale dynamical parameters, much like Λ_{AFM} . Interestingly for the C_{1D} strain, two nanodomain sizes are evident, with one value being close to C_0 ($d > \xi_{1D}^H$) and the other being very close to C_1 ($d < \xi_{1D}^L$) and C_2 . Also, apart from the intermediate length scale diffusion regime, the ratio D_L/D_H is very close to that for C_1 . It is therefore intriguing to suggest that the C_{1D} strain is in an

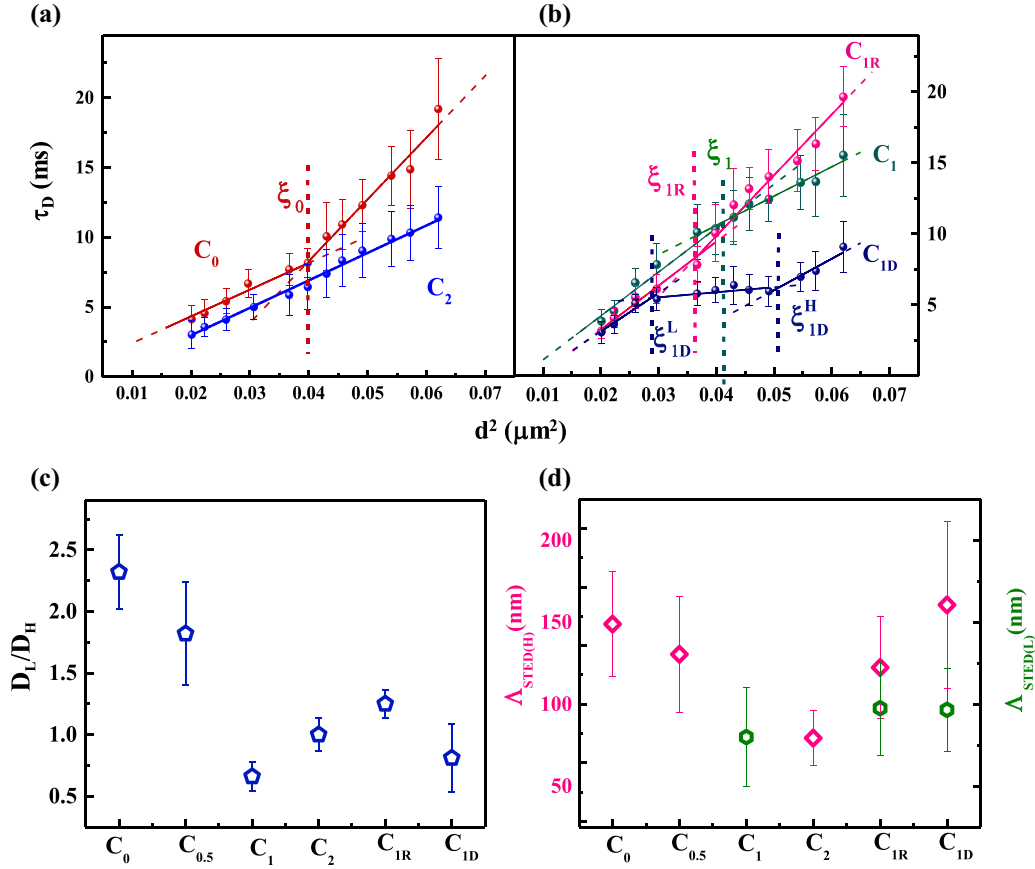


FIG. 3. Transit time τ_D dependence on the focal spot area d^2 for (a) C_0 , C_2 ; and (b) C_1 , C_{1R} , and C_{1D} strains. Nile red was used as a marker to label the bacterial membrane. τ_D values were extracted using Eq. (1). The d where there is a change in the slope of the plots is called the crossover length scale, represented by ξ_x . Here a subscript represents the concentration of Colistin. A superscript is used for C_{1D} strain as it has two crossover points representing the high/low (H/L) d region. (c) D_L/D_H values extracted from the slope values (Table S1 in the SM [23]) for various strains. For $d < \xi$, D_{eff} has been termed D_L and for $d > \xi$, it has been termed D_H . (d) Nanodomain size extracted using the equation $\Lambda_{\text{STED}} = \sqrt{8D_{\text{eff}} \ln 2 [t_0]}$ for various strains. Each data point is derived from measurements collected over several bacteria, as detailed in the Appendix, which provided the statistical deviation from the mean values.

intermediate state of evolution and membrane compositional reorganization, and our membrane nanoscale dynamic probe is able to capture the existence of this transitional state of the bacterial cells. To further probe the dynamical nature of these membrane nanodomains and their reorganization due to the Colistin-mediated resistance evolution, we explore nanoscale temporal fluctuations of the lipids using a well-known statistical method for estimating the non-Gaussian parameter (NGP) for number fluctuations.

D. Nanoscale temporal fluctuation reveals dynamical membrane reorganization due to Colistin

While the SV-FCS results presented above reveal the presence of lipid nanodomains and their subtle reorganization due to the emergence of Colistin-mediated phenotypic resistance, insight into the microscopic process leading to these observations is incomplete. Probing higher-order fluctuation correlations in physical systems has typically revealed additional insight into the underlying microscopic process driving such fluctuations. A non-Gaussian parameter is one such parameter typically used to quantify higher-order fluctuation correlation, and it can be associated with the pres-

ence of dynamical heterogeneity in disordered systems such as glasses, supercooled liquids, and model biomembranes [41–43]. While typically particle displacement fluctuation correlation is calculated in most cases, here we have estimated α_2 from the fluctuation of N with respect to $\bar{N}$, within d , N being the number of fluorescent lipid molecules, and $\bar{N}$ being the average number. The NGP parameter α_2 can be defined as [18]

$$\alpha_2 = \frac{1}{3} \frac{\langle (N - \bar{N})^4 \rangle}{\langle (N - \bar{N})^2 \rangle^2} - 1. \quad (5)$$

Figure 4(a) shows typical data for fluctuations in N for two fixed values of d for C_0 and α_2 captured at $d = 140$ nm for C_1 and C_2 . While for larger d the deviations appear to be random and hence Gaussian for the C_0 strain, the data captured at smaller d show a significant presence of large deviations in N from $\bar{N}$. This suggests the presence of a certain degree of cooperativity of the diffusing lipids at the nanoscale in the C_0 membranes. It is very interesting to note from Fig. 4(a) for C_2 that the sharp fluctuations significantly reduced compared to C_0 and C_1 at the same value of d .

These observations are summarized in Fig. 4(b) in the form of the variation of α_2 with d^2 . In particular, it can be seen in

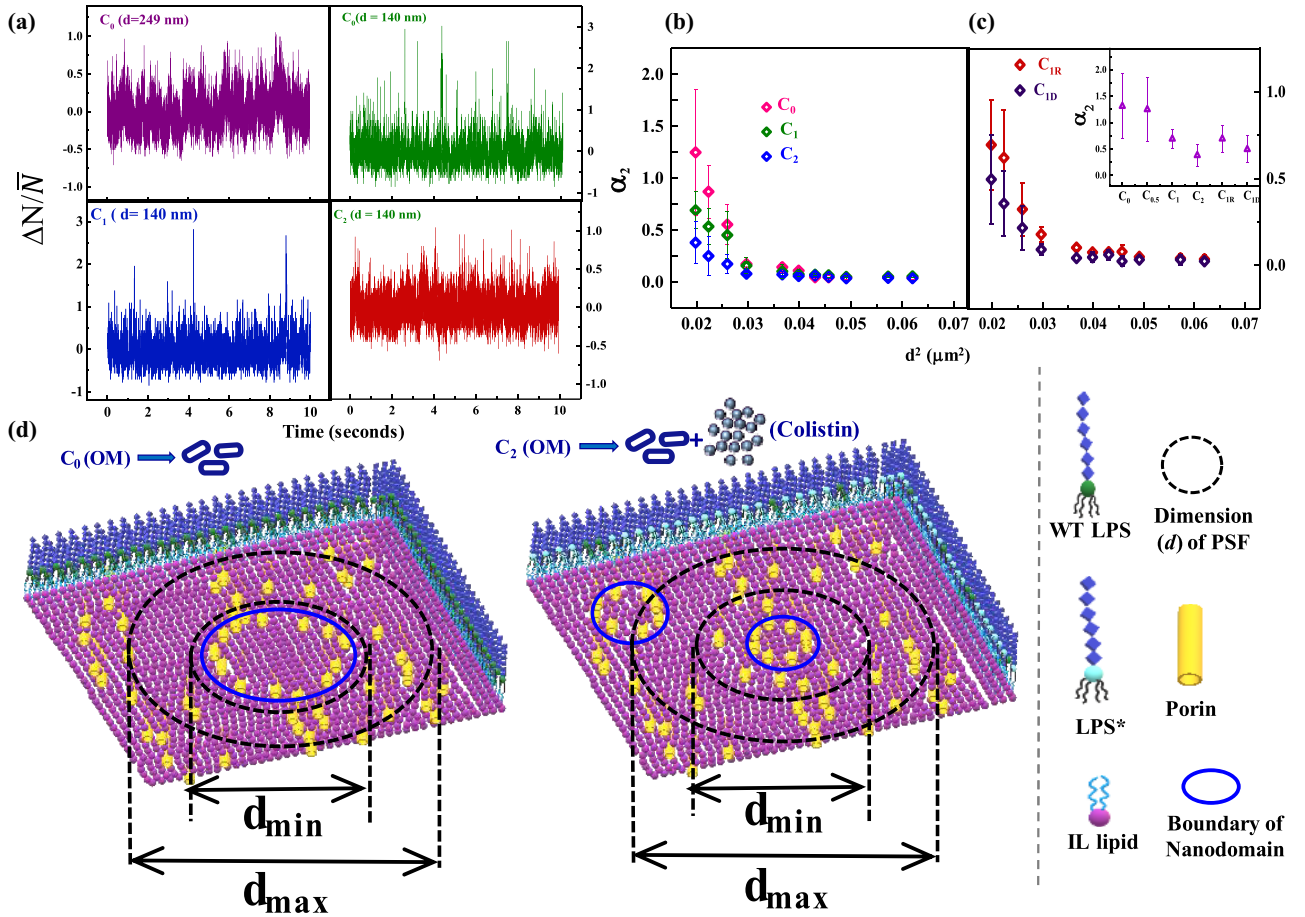


FIG. 4. (a) The graphs depict the variation of $\Delta N/\bar{N}$ ($\Delta N = N - \bar{N}$) with collection time, with the top panels representing values from the C_0 membrane with $d = 249$ and 140 nm probing scales, and the bottom panels showing data from C_1 and C_2 membranes with $d = 140$ nm. (b) Dependence of α_2 on the focal spot area d^2 . It is clear from the figure that α_2 has reached the maximum value for the samples C_0 (≈ 1.25), $C_{0.5}$ (Fig. S10), and C_1 , whereas it does not increase so much for C_2 . Part (c) shows the trend of α_2 against spot area d^2 for C_{1R} and C_{1D} strains. The inset shows the plot of α_2 at the smallest spot size for all the strains, and C_{1R} may indicate the reduction in the dynamical heterogeneity for C_2 strain. Part (d) depicts the outer membrane (OM) of C_0 (left) and C_2 (right) with nanodomains encircled by a solid black line. The region encircled with dotted black lines shows the minimum PSF ($d_{\min}$) and the largest PSF size ($d_{\max}$) used in the (SV)-FCS experiments. The moieties of the OM have been labeled, including the WT LPS found predominantly in C_0 , modified LPS (LPS*), where the negative charge of lipid A had been neutralized by the incorporation of positively charged moieties, inner leaflet (IL) lipids, and porins. The C_0 nanodomains were comparable to $d_{\min}$ whereas the C_2 nanodomains were much smaller than $d_{\min}$. So, $\Delta N/\bar{N}$ captures non-Gaussian fluctuations in C_0 OM at $d = d_{\min}$, thus capturing hindered diffusion prominently posed by the nanodomain. Δ_{STED} being much smaller than $d_{\min}$ in C_2 OM, the non-Gaussian fluctuations of the diffusing species were not a prominent contributing factor as compared to the freely diffusing IL lipids.

Fig. 4(b) that the difference in α_2 values at smaller d^2 between C_0 , C_2 is quite significant. It is also visibly smaller in C_1 compared to C_0 . Similarly, in Fig. 4(c) we see a clear difference between C_{1R} and C_{1D} . To summarize these observations, we show in the inset of Fig. 4(c) the variation of α_2 for various strains for a fixed d^2 . It is quite fascinating to observe that the α_2 values reach a minimum for C_2 and then rise again for C_{1R} , very similar to trends observed across multiple parameters as discussed earlier. To understand the underlying reasons for these observations, we consider the schematic in Fig. 4(d). Essentially, there is an interplay of nanoscale cooperative motion and the size of the probe region which is reflected in the observed large deviations in $\Delta N/\bar{N}$ and hence enhanced values of α_2 compared to a scenario where the deviations

are normally distributed. In particular, we illustrate how the relative values of Δ_{STED} or even Δ_{AFM} compared to $d_{\min}$ determine the sensitivity to detection of fluctuations. Clearly, for large values of d , in particular around $d_{\max}$ ($=249$ nm), which is much larger than any detected nanodomain sizes, none of these cooperative lipid motions can be detected. On the other hand, for d values around $d_{\min}$, we see that for many strains $\Delta_{\text{STED}} \leq d_{\min}$ ($=133$ nm) and hence in this regime SV-FCS becomes very sensitive to such dynamic cooperative lipid domains and shows up in the form of large deviations in $\Delta N/\bar{N}$. This difference in Δ_{STED} values between C_0 and C_2 is the reason for the observed variation in α_2 and is illustrated in Fig. 4(d) for C_0 and C_2 . In fact, we predict that further reducing $d_{\min}$ would make it possible to detect fluctuations in C_2 .

III. DISCUSSION

Our recent studies [22] revealed Colistin dosage dependence changes in *E. coli* membrane lipid diffusion and surface roughness. Interestingly, in resistant *E. coli* cultures (NCTC 13846) having highly genotypic strains, no significant effects of Colistin were observed even at a dosage of almost 20 times that of the susceptible *E. coli* cells, which show membrane dynamics changes. In that particular study, the resistant strain was known to have modified LPS with the lipid A negative charge neutralized [44,45]. Here we have extended our earlier study and tracked changes in nanoscale membrane dynamics and morphology with development and reversal of phenotypic resistance in *E. coli* cultures. We identify physical signatures of a noninheritable mechanism of the evolution of Colistin resistance in *E. coli*, with a specific emphasis on activation of immediate adaptive signaling response mediated by changes in membrane composition. To the best of our knowledge, this is the first study that correlates and quantifies the underlying similarity between trends observed through various biochemical assays on *E. coli* cultures which develop and reverse phenotypic resistance with single-cell bacterial membrane nanoscale morphology and dynamical parameters.

Our findings present diverse pieces of evidence demonstrating how bacteria may adapt to the selective pressure imposed by Colistin exposure. The resistance feature was lost when Colistin was withdrawn, thereby documenting the presence of previously unreported plasticity in adaptation to bactericidal antibiotics like Colistin. We acknowledge the existence of well-documented genetic (*de novo*) mechanisms associated with Colistin resistance, typically driven by mutations in key genes, e.g., *mur*, involved in lipid A modifications. Our observations, which included reversible changes in membrane fluidity, permeability, organization, and gene expression changes, shed light on the AMR landscape beyond the genetic framework, which can explain rapid phenotypic changes to antibiotic pressure. Our study reveals the presence of additional mechanisms that involve sensing and adapting to changes in the membrane brought about by Colistin, by specific two-component systems PhoPQ and PmrAB, which then alter the expression of genes involved in lipid A modification, thereby leading to the emergence of Colistin-resistant *E. coli* strains. The observations based on gene expression changes recorded with Colistin treatment revealed up-regulation of genes coding for the PmrAB two-component system. Reversal of *pmrAB* gene expression was observed after Colistin withdrawal leading to the restoration of Colistin sensitivity (ColES) after a few generations indicative of gradual loss of resistance triggered by accumulation of modified lipid A. These findings also indicate the presence of a continuous positive feedback loop in the presence of Colistin to maintain a Colistin-resistant state.

Recent studies have shown that PmrB binds ferric iron [46,47] and modulates changes in the transcription of its targets through its response regulator PmrA, including *phgP* and *ugd*, leading to LPS modification, which drives Polymyxin and Colistin resistance. Interestingly, transcription of PmrA-activated genes can also be induced by growth in low magnesium through a process that requires another two-component system, PhoPQ. PhoPQ TCS transduces the low

magnesium signal to the PmrAB TCS by using PmrD as a mediator. Given that Colistin displaces Mg and Ca ions from lipid A, it also activates the PhoPQ system, thereby further driving resistance emergence. These results align with our findings, where the up-regulation of *pmrAB* and *phoPR* TCS in strains eventually exposed to 1 μg per mL and 2 μg per mL Colistin was recorded, which was lost when the drug is withdrawn, as after some passages the bacteria loses the changes driven by TCS activation due to complete replacement of modified lipid A with newly synthesized lipid A, thereby reintroducing sensitivity towards Colistin. Probing higher-order correlations in lipid dynamics revealed signatures of lipid cooperative dynamics and dynamic heterogeneity which was somewhat anticorrelated with the extent of resistance in the strains. The magnitude of the non-Gaussian parameter, α_2 , also seems to be minimal at the lowest nanodomain sizes, which also happens to be for strains displaying maximum antimicrobial resistance. The changing nanodomain sizes seem to determine the extent of cooperativity and hence the value of α_2 . Our study thus reveals the subtle pathway and feedback mechanism for the emergence of antimicrobial resistance by probing membrane nanoscale organization and lipid dynamics. Evidence for cooperative molecular motion in supercooled liquids and glasses upon approaching the critical temperature has been widely reported [48–51], and it is intriguing to note similar lipid dynamics in bacterial cell membranes. The exact reason for the close connection between the phase-separated LPS domain in the outer leaflet of the outer membrane and the dynamical nanodomains observed for lipids in the inner leaflet needs to be investigated. Overall, our studies show that while antimicrobial resistance typically has genetic determinants, immediate signatures of the emergence of antibiotic resistance can be captured using a high resolution and sensitivity biophysical interrogation of membranes, which can give a potentially faster readout of potential AMR development, thus ushering in a new paradigm for detection of AMR.

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APPENDIX

The *E. coli* bacteria used in these studies were obtained from the American Type Culture Collection (ATCC 10536) and were cultured overnight in a Luria-Bertani (LB) nutrient medium for 12 h. Poly-L-lysine (PLL) purchased from Sigma Aldrich was used to immobilize the cells during imaging. Colistin sulfate, the antibiotic used in the studies, was also obtained from Sigma Aldrich. Nile red, a fluorescent marker, was used for labeling and was procured from Sigma Aldrich as well. Nile red was employed for imaging and FCS.

1. Bacterial culturing

To initiate the primary culture of the cells, a sterile loop or inoculation needle was used to pick up a portion of cells from a single colony on an LB Agar plate, which was then inoculated into 5 mL of LB medium. The culture was incubated overnight at 37 °C with continuous shaking with an rpm of 180 in the incubator. After 12 h, when the culture reached saturation phase, it was termed C_0 , which is the acronym for wild type. 1% aliquot of this sample of C_0 was inoculated in an LB medium containing 0.5 µg/mL Colistin and cultured in the incubator in similar conditions. After the bacteria grew for 12 h, the strain was termed $C_{0.5}$. $C_{0.5}$ strain was serially passaged in 1 µg/mL Colistin and subsequently in 2 µg/mL concentrations; the strains after growing in that antibiotic stress will be termed C_1 and C_2 , respectively. MIC of Colistin for pristine *E. coli* was determined to be 2 µg/mL using the Broth Micro dilution assay, as discussed below.

2. MIC determination

MIC refers to the lowest concentration of an antibacterial agent, expressed in milligrams per liter (mg/L) or (µg per mL) that can prevent the visible growth of a specific strain of an organism under carefully controlled *in vitro* conditions [52]. The growth curve of *E. coli* 10536 was established by monitoring absorbance at a 620 nm wavelength. The optical density OD_{620} acted as an indicator of bacterial cell growth using a spectrophotometer (Tecan, The Infinite 200 PRO). 1% inoculum of *E. coli* 10536 was introduced to the growth medium, and the cultures were then incubated at 37 °C with continuous shaking (180 rpm) in the 96 well plates. Data were collected continuously for 20 h, with data collection occurring at 15-min intervals. The OD_{620} values were plotted, taking into account the OD data at saturation, for different concentrations of Colistin, and represented as a function of Colistin concentration. Based on the data presented in Fig. S1 of the SM [23], the MIC values for C_0 and $C_{0.5}$ were the same. However, a notable transition occurred for the C_1 strain, whereby the MIC reached 16 µg/mL, and this MIC value remained the same for the C_2 strain. Due to the observed changes in the MIC measurements for the C_1 strain, we decided to restrict our measurements to concentrations up to 2 µg/mL, which is C_2 strain.

3. *E. coli* labeling protocol

All Fluorescence Correlation Spectroscopy (FCS) and STED-FCS measurements with Nile red were conducted at an OD of 0.4–0.6. After reaching the saturation stage, the strains C_0 , $C_{0.5}$, C_1 , C_{1D} , C_{1R} , and C_2 were aliquoted into an LB medium with the same/no amount of Colistin as was present in the mother culture, to obtain an initial secondary inoculum containing 1% bacterial concentration compared to the saturated culture. The inoculum was then incubated at 37 °C in a shaking incubator at 180 rpm rotation speed until the culture reached an OD of 0.4–0.6. To 1 mL of the culture, 1.5 µg/mL Nile red was added such that the dye concentration becomes 1.5 µg/mL or 4.71 µM. The solution was mixed properly. The solution was then incubated at 37 °C in the incubator at an rpm of 220 for 5 min. Subsequently, the solution was centrifuged at 3600 rpm for 5 min. The bacteria settled down,

and the supernatant containing unlabeled dye was discarded. To the precipitated bacteria, 1 mL PBS (phosphate buffered saline) was added and mixed. The culture was again spun down, and the supernatant was discarded. Finally, the pellet was resuspended in 500 µL of PBS. Separately, cover slip washed with ethanol and dried was dipped completely in 0.01% w/v of Poly-L-lysine (PLL) solution. The cover slip remained dipped for an hour and then was washed with DI water. The Nile red-labeled bacterial cells were spread on the PLL-coated coverslip and gently dispersed using a sterile tip. These cells were then washed with PBS to remove any unbound bacteria. The cover slip containing the immobilized bacteria was then attached to a glass slide having a hole of around 1 cm dimension at the center. The cover slip was attached using double-sided tapes (Bioscience Tools). Due to the hole, a well was created above the cover slip having a volume of around 250–300 µL. The well was then filled with PBS to keep the bacteria always in a buffered environment during the microscopic measurement.

4. EtBr uptake assay

The serially passaged strains were cultured as described previously. The cultures present in the saturation phase were regrown to the logarithmic phase of 0.4–0.6 OD as described in Methods, subsection C. The cells were washed in PBS and their OD were measured. The concentrations of the cultures were made identical by diluting the higher OD cultures. Triplicates of each sample were added in a light-blocking flat-bottom 96-well plate. To 100 µL of the added cultures, 4 µg/mL of Colistin was added. Subsequently, 1 µg/mL of EtBr was added and the fluorescence was measured after 60 min after EtBr addition. The background signal was measured from PBS containing EtBr and subtracted from the sample fluorescence.

5. RT PCR Protocol

The qRT-PCR primers were meticulously designed by targeting a specific sequence within the gene of interest. Utilizing the Primer3 primer design software, primers were crafted to be approximately 18–24 nucleotides in length, possessing a GC (guanine-cytosine) content ranging from 40% to 60%, and exhibiting similar melting temperatures (T_m), typically around 60 °C, for both forward and reverse primers. The forward and reverse primers were carefully designed to avoid secondary structure formation, self-complementarity, and mutual binding to each other.

6. Atomic force microscopic imaging

Before the experiments, the cover glass slides were cleaned by sonicating in ethanol for 30 min. These were then blown dry using nitrogen purging. These were then dipped in 0.01% (w/v) Poly-L-Lysine (PLL) for 1 h. Thereafter, the cover glass slides were washed with ultrapure water and dried. As described in Methods subsection C, the bacteria were cultured and prepared. However, instead of resuspending the bacterial pellet in 500 µL PBS, in this case the pellet was resuspended in 300 µL PBS. From the bacterial suspension, 4 µL aliquot was drop-casted onto the cover glass slide. The bacterial cells were spread on the PLL-coated coverslip and gently dispersed using

a sterile tip. The sample was dried in an ambient condition for 10 min to observe a white patch of bacterial monolayer. These cells were then washed with ultrapure water to remove any unbound bacteria. The sample was then dried for 1 h to remove any water droplet which might interfere in AFM imaging in a dried condition. The sample was then imaged from the point of drying for a duration of 6 h. This was followed consistently for all the bacterial samples. Atomic force microscopy (AFM) measurements were performed using a Park System (NX 100, Korea) in a noncontact mode. A cantilever with a force constant of 5 N m^{-1} , a free amplitude of 160 kHz, and a tip radius of curvature 8 nm was used for the measurements. AFM images were acquired using the HQ:NSC14/AI BS probe (MicroMasch) with a set point of approximately 15–20 nm. The noncontact mode of AFM allowed for imaging the surface of the samples without physical contact between the cantilever tip and the bacteria. However, acquiring phase images of a section of the bacterial outer membrane was implemented using the tapping mode. The scanning frequency was kept between 0.7 and 1 Hz. Depending on the scan area, the proportional and integral gains were tuned to obtain good images. The images were flattened to remove shadow artefacts and scanner drift using XEI software. Any high-frequency tip artefacts in the image were removed using Fourier filtering using the XEI software.

7. Stimulated emission depletion fluorescence correlation spectroscopic measurements

For imaging, FCS, and STED-FCS nanoscopy measurements, a STEDYCON optical setup (Abberior Instruments

GmbH) integrated with an SP5 Leica microscope was utilized. The bacterial samples were placed on the $100\times$ objective of the microscope. Bacterial samples labeled with Nile red were excited using a 561 nm laser, and the emitted fluorescence was detected using a detector with a range of 575–625 nm. The STED measurements employed a depletion beam at a wavelength of 775 nm, which was generated using a spatial light modulator. This depletion beam created a doughnut-shaped point spread function (PSF) at the imaging plane, thereby reducing the effective PSF at the imaging plane. The PSF calibration was carried out at various STED 561 powers using calibration beads provided by Abberior Instruments GmbH, along with a standard DOPC bilayer labeled with Nile red. The STED powers were systematically adjusted, ranging from 0 mW at 0% to 159 mW at 100%, thereby modulating the size of the central maxima of the doughnut-shaped pattern. The calibration details can be found in the Supplemental Material [23], providing a description of the calibration process. Both FCS and STED-FCS measurements were performed for a duration of 12–20 s per measurement. An average of five to six points on each bacterium were sampled, with a total of 10–15 bacteria analyzed during one entire STED-FCS experiment of a particular sample. At least 15 FCS profiles were used to plot a single data point with statistical error bars in the STED-FCS profiles. Time-correlated single photon counts were collected using the Avalanche Photodiode (APD) of the Abberior system, which was interfaced with Pico-quant Symphotime software. The autocorrelation data were fitted to a single-component two-dimensional diffusion model as described in the Supplemental Material [23].

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