Supporting Information For

Rapid Electrochemical Monitoring of Bacterial Respiration for Gram-Positive and Gram-Negative Microbes: Potential Application in Antimicrobial Susceptibility Testing

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CONTENTS

1. Experiments.

1.1. Colony count of the bacterium.

The colonies were counted and cfu/mL were determined using the following relation.

$$cfu/mL = \frac{No.of\ colonies\ \times dilution\ factor}{Volume\ of\ sample\ on\ plate\ (ml)} \quad \text{--- (1)}$$

1.2. Development of electrochemical cell configuration to monitor the respiration of bacteria.

The electrochemical experiments were performed in three-electrode cells, employing platinum wire as a counter electrode, Ag/AgCl (3 M KCl) as a reference electrode (Metrohm, India) and ITO or *l*-lysine functionalized cerium oxide nanoparticle coated ITO (L-CeONP/ITO) as working electrode.

1.2.1. Preparation of various solutions

The solutions of $K_3Fe[CN]_6$ and $C_6H_{12}O_6$ with varying concentrations of (1 to 10 mM and 10 to 50 mM respectively) were prepared in fresh phosphate-buffered saline (PBS) [50mM, pH 7.0] for cyclic voltammetry (CV) measurement. The stock solution of ciprofloxacin, cefixime, and amoxycillin were prepared in saline form powdered stocks and used in the same day, this stock solution was further diluted to 2 µg/µl for susceptibility testing.

1.2.2. Preparation of Bacterial cell for electrochemical assay

The pure cultures of *E. coli* and *B. subtilis* were grown overnight at 35±1°C in Luria Broth. To prepare a stock solution, the *E. coli* and *B. subtilis* were transferred to 0.9% saline via centrifugation at 3000 rpm and redispersion. This stock solution was further diluted to prepare different concentrations of *E. coli* and *B. subtilis* for electrochemical testing.

1.2.3. Measurement of live bacterial concentration using electrochemical measurement

The ITO or L-CeONP was utilized as a working electrode for electrochemical testing. For the preparation of the working electrode, ITO (with area 0.77 cm^2) was cleaned with DI water and warm acetone; to remove impure residue from the substrate. For electrochemical measurement of

live bacterial respiration, 250 µl of bacterial cells in 0.9% saline (*E. coli* or *B. subtilis*) at varying dilution was charged onto electrolyte containing $K_3Fe[CN]_6$ (5 mM) as an electron acceptor and $C_6H_{12}O_6$ (10 mM) as a carbon nutrient in freshly prepared PBS, were then analyzed using CV within the applied DC voltage of -0.4 to 0.8 V at a scan rate of 100 mV s^{-1} using three-electrode system equipped with ITO or L-CeONP as working, Ag/AgCl (3 M KCl) as reference and Platinum wire as counter electrode. The anodic peak current of the CV plot was recorded for various dilutions of bacterial cell culture in 0.9% saline to plot the calibration curve of the anodic peak current of $Fe(CN)_6^{3-}$ vs concentration of bacteria in cfu/mL and same was validated with OD_{600} measurements. Prior to calibration, the concentration of electron acceptor $K_3Fe[CN]_6$ from 1 to 10 mM was varied in freshly prepared PBS containing 10 mM $C_6H_{12}O_6$ and the pH was optimized by adjusting the pH of PBS from (6, 6.5, 7 and 7.5) in solution containing 5 mM $K_3Fe[CN]_6$ (Figure S3). The carbon nutrient $C_6H_{12}O_6$ of varying concentration from 10 to 50 mM were tested in freshly prepared PBS containing 5 mM $K_3Fe[CN]_6$ at pH 7.

1.3. Antibacterial Susceptibility testing (AST) of bacteria through electrochemical testing using ITO

For the following experiments, testing concentration of *E. coli* (OD_{600}=0.1; 3.5×10^6 cfu/mL) and for *B. subtilis* (OD_{600}=0.3; 1.8×10^{13} cfu/mL) were used. The *E. coli* and *B. subtilis* in 0.9% saline have interacted with amoxycillin, ciprofloxacin, and cefixime of identical dose (2 µg/µl) individually. Then at different time intervals (0 m, 15 m, 1 h, 2 h, and 18 h) the bacterial cells were recovered via centrifugation and resuspended in an equal volume of saline to remove antibiotics solutions. From this, 250 µl of saline suspended with *E. coli* or *B. subtilis* was charged onto 30 ml of the electrolyte solution in 3-electrode cell and the electrochemical measurement was performed against Ag/AgCl as a reference electrode in $K_3Fe[CN]_6$ and $C_6H_{12}O_6$ solution of an electrolyte. For each electrochemical measurement of the bacterial cell, the anodic peak current of CV with the control sample (i.e., bacterial cell without antibiotics) was also measured electrochemically under identical conditions. The difference in anodic peak current of control and anodic peak current of the antibiotic interacted bacterial cell was observed. To validate the observation made by electrochemical testing, dose of ciprofloxacin, cefixime and amoxycillin (=2 µg/µl) with *E. coli* (OD_{600}= 0.1) were maintained analogs to the electrochemical testing: a traditional plate count method was performed for *E. coli* cells at similar time interval of

antibiotic interaction to that of electrochemical testing (0 m, 15 m, 1 h, 2 h, and 18 h) and quantification *E. coli* in cfu/mL was performed according to section ***1.1***.

1.4. Time-lapse microscopy setup

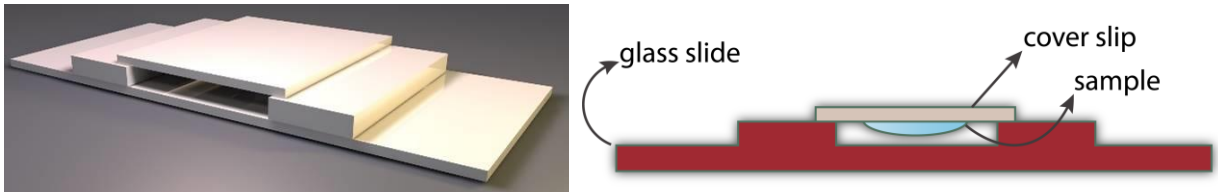

Figure S1| Custom build hanging drop sample loading setup used for time-lapse microscopy measurement.

The Time-lapse microscopy experiments were performed at ambient temperature with Olympus Cx23 operating in a bright field, equipped with (Magcam DC). For this experiment, *E. coli* and *B. Subtilis* cells grown overnight in Luria Broth at 35±1°C were transferred to sterile Luria broth individually, to a concentration of *E. coli* (OD_{600}=0.1) and *B. subtilis* (OD_{600}=0.3) like that of electrochemical AST in section ***1.3***. The custom build setup as shown in Supplementary figure 1 was prepared on a glass slide, the samples containing cell dispersion with antibiotics (2 µg/µl) were loaded onto the setup by placing a 5 µl drop on the coverslip and hanging it as shown in the Supplementary figure 1; the time between addition of antibiotic and loading is kept short is possible (ca. 5s). The time-lapse measurements were observed using 40× objective, at different time point bacterial cells were tracked using Manual track plugin in Fiji. The measurements are recorded as their mean total velocity. And for automated measurements algorithm in the mosaic particle tracking plugin in Fiji was implemented.

1.5. Atomic Force Microscopy (AFM).

The effect of antibiotics towards *E. coli* cells at 0 h and 2 h were monitored using AFM, *E. coli* cell suspension was interacted and separated from antibiotic solution as mentioned in section ***1.3***. For AFM analysis the glass substrate was cleaned with warm acetone and subsequently immersed in ethanol/HCL (70:1 volume ratio) solution for 2 h at 40°C, then it was cleaned in ethanol and rinsed with DI water. The clean substrate was sterilized under UV light for 30 min and subsequently air-dried, *E. coli* cell suspension was fixed onto the glass substrate by air drying in laminar flow, to track the bacterial cells during AFM measurements 0.5% Toluidine

Blue-O was stained on to the smear. The AFM measurements were performed with NTEGRA prima AFM (NT-MDT, Russia) in tapping mode using NSG03 silicon cantilever (N-type, Antimony doped, PtIr reflective coating). The resonant frequency of the tips was 47-150 kHz and applying a force constant of 0.35-6.1 N/m. The obtained images were analyzed using NOVA SPM and Gwyddion[1].

1.6. Measurement of AST using l-lysine coated cerium oxide nanoparticles(L-CeONP) modified ITO(L-CeONP/ITO).

1.6.1. Preparation and characterization of L-CeONP

l-lysine coated ceria nanoparticles (L-CeONP) were prepared by chemical precipitation reaction. In this procedure, 10 ml of DI water was maintained at 40°C, followed by the addition of 400 µl of *l*-lysine(1M) and 200 µl of cerous nitrate hexahydrate (1M). Then, 500 µl of ammonia(1M) was added dropwise into the reaction mixture and the reaction continued for 2 h. The particles were washed with ethanol and dried in an oven at 60°C. Also, Pluronic coated cerium oxide (P-CeONP) and uncoated CeONP was prepared using a similar procedure. The L-CeONP were characterized using powder X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM).

1.6.2. Measurement of Antibacterial properties of Cerium oxide nanoparticles (CeONP)

The antibacterial activity of CeONP, P-CeONP, and L-CeONP was monitored by measuring the change in turbidity (OD_{600}) of Luria broth inoculated with only *E. coli*, CeONP & *E. coli*, P-CeONP & *E. coli*, and L-CeONP & *E. coli,* for this experiment *E. coli* were obtained as per section ***1.2.2.*** The measurements were performed in triplicates and the response is monitored as a change in OD_{600} values.

1.6.3. AST of E. coli using L-CeONP/ITO through electroanalysis

The ITO substrate was cleaned with warm acetone, methanol, and DI water. Then, an aqueous solution of L-CeONP (=1µg/µl) was prepared in fresh PBS buffer (50 mM, pH=7.0), after ultrasonication for 15 m, 20 µl of this solution is drop-casted on the edge of ITO (with area $0.77/cm^2$) in addition it was allowed to dry at 40°C. This concludes the preparation of L-CeONP/ITO for the use in EAST as the working electrode. All the procedures and set up for the

electrochemical AST are like that of section ***1.3*** except the change in the working electrode from ITO to L-CeONP/ITO.

1.7. Testing B. subtilis for Endospores.

Schaeffer-Fulton method[2] and Heat kill assay are used to find out endospores in *B. subtilis*. The sample of *B. subtilis* for interaction with antibiotics was prepared like the growth conditions for EAST. Schaeffer-Fulton method was implemented after the transfer of *B. subtilis* from nutrient-rich LB broth to saline and at 15 min of interaction with ciprofloxacin and cefixime in saline. The Heat-Kill assay was performed by treating the *B. subtilis* cells at 80℃ for 20 min and streaking it on LB plates[3], *B. subtilis* dispersed in saline were directly heat-treated, whereas the ciprofloxacin and cefixime treated cells were centrifuged to remove antibiotics and again dispersed in sterile saline(0.9%) which is then streaked on to LB plates. To further confirm the Heat-Kill assay Fluorescence microscopy measurements were done; the heat-treated *B. subtilis* cells were stained with acridine orange (1 mg/mL, in PBS: pH 7.4) and ethidium bromide (1 mg/mL, PBS: pH 7.4) to confirm the viability.

1.8. Statistical analysis.

All the experiments were repeated at least 3 times ($n \geq 3$), ANOVA statistics were performed using OriginPro 8.5 and GraphPad Prism 6.

2. Results and Discussions.

2.1. Kinetic analysis

The following is the equation form Randles-Sevcik equation, which here is used to calculate the diffusion coefficient,

$$I_p = (2.69 \times 10^5)\, n^{3/2}\, A\, \mathbf{D}^{1/2}\, C\, \nu^{1/2} \quad \text{---(2)}$$

where n represents the number of electrons involved or electron stoichiometry(in our case n=1), A= 0.77/cm^2 is the area of the working electrode, C is the concentration of redox species(5mM), **D** is the diffusion coefficient and ν is the scan rate(= 100 mV/s).

The following equation is from Lavrion Model used here to calculate the electron transfer rate.

$$k_s = \frac{mnF\nu}{RT} \quad \text{---(3)}$$

where m represents peak to peak separation, F is the faraday constant(96485.3 C/mol), ν is the scan rate(100 mV/s), n is the number of transferring electron(= 1), R is the gas constant(8.314 J mol^{-1} K^{-1}) and T is room temperature(=298 K). The $\mathbf{k_s}$ values indicate electron transfer between electrode and moiety near an electrode in the presence of gram-negative bacteria.

2.2. Exponential Fit for decay calculation.

Using a one-phase exponential decay function with time constant parameter, the electrochemical response of *E. coli* was fitted using the following equation for 0-18 h response curves.

$$y = y_0 + Ae^{-x/t} \quad \text{--- (4)}$$

where y_0 is offset, A is amplitude and t is the time constant.

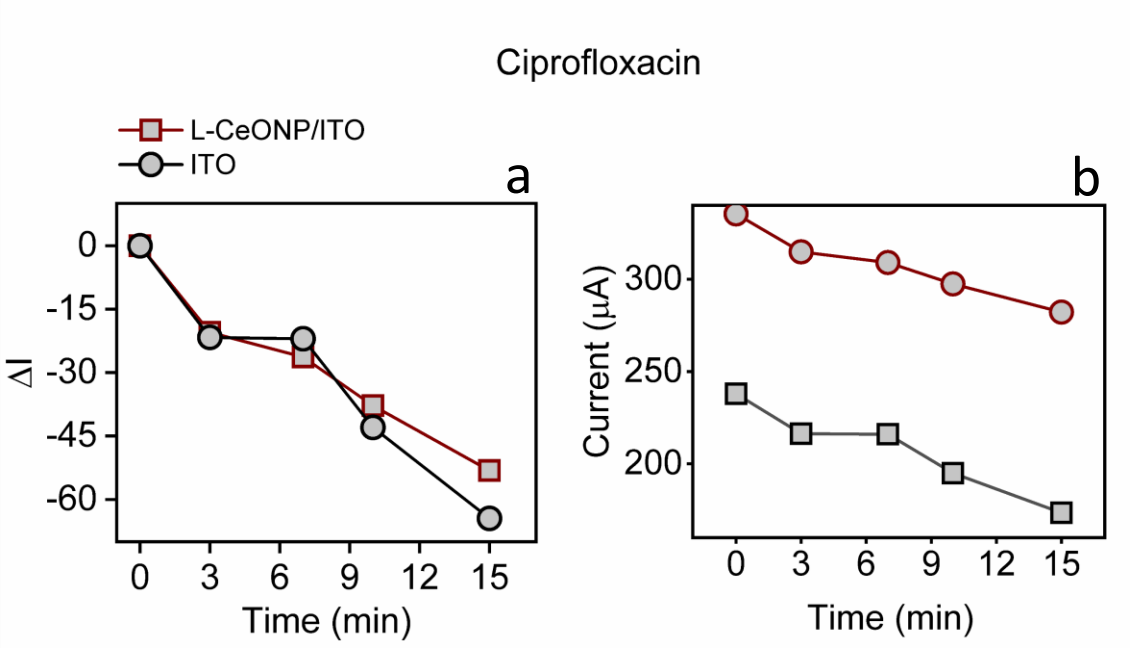

Figure S2| a, Difference current plot of *E. coli* EAST response within 15 min of interaction with Ciprofloxacin, compared with L-CeONP coated ITO working electrode and ITO working electrode. **b**, shows the enhanced current response obtained.

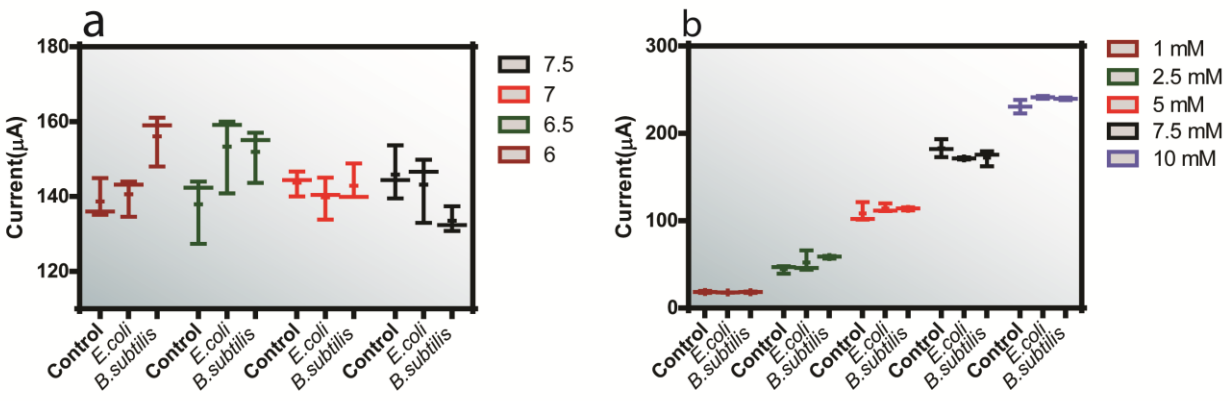

Figure S3| a, shows the electrochemical response of *E. coli* and *B. subtilis* at different pH with only 5 mM $K_3Fe[CN]_6$. b, plots the electrochemical response of *E. coli* and *B. subtilis* at different concentrations of $K_3Fe[CN]_6$.

Discussion for **Figure S3**.

Prior to the optimization studies using carbon source ($C_6H_{12}O_6$), observation of *E. coli* and *B. subtilis* electrochemical response with different pH (6 - 7.5) and by varying $K_3Fe[CN]_6$ concentration from 1-10 mM was performed. The results of which are plotted in Figure S3 a and b. Out of these measurements, we selected a 5 mM concentration of $K_3Fe[CN]_6$ at pH=7 for the observation on the electrochemical response of the bacterium in the presence of carbon source.

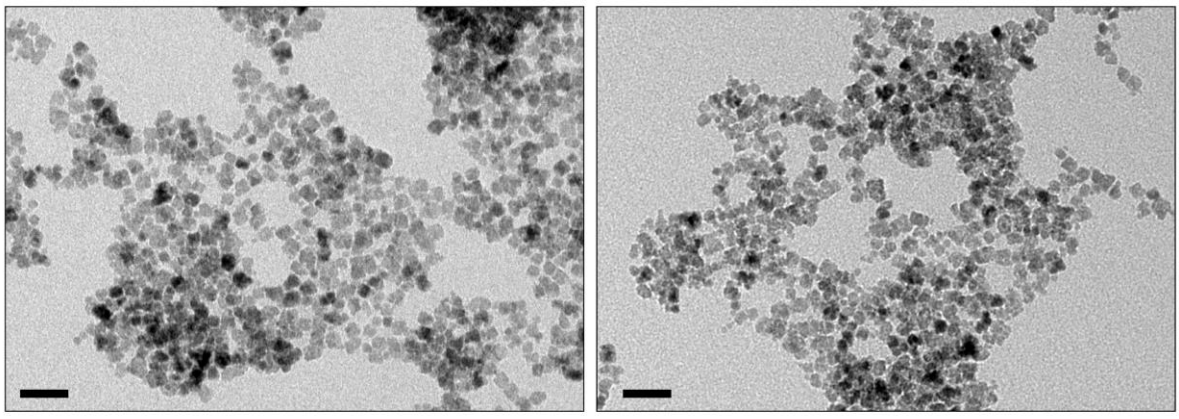

Figure S4| Transmission electron microscopy images of *l-lysine* coated cerium oxide nanoparticles. (scale=20 nm)

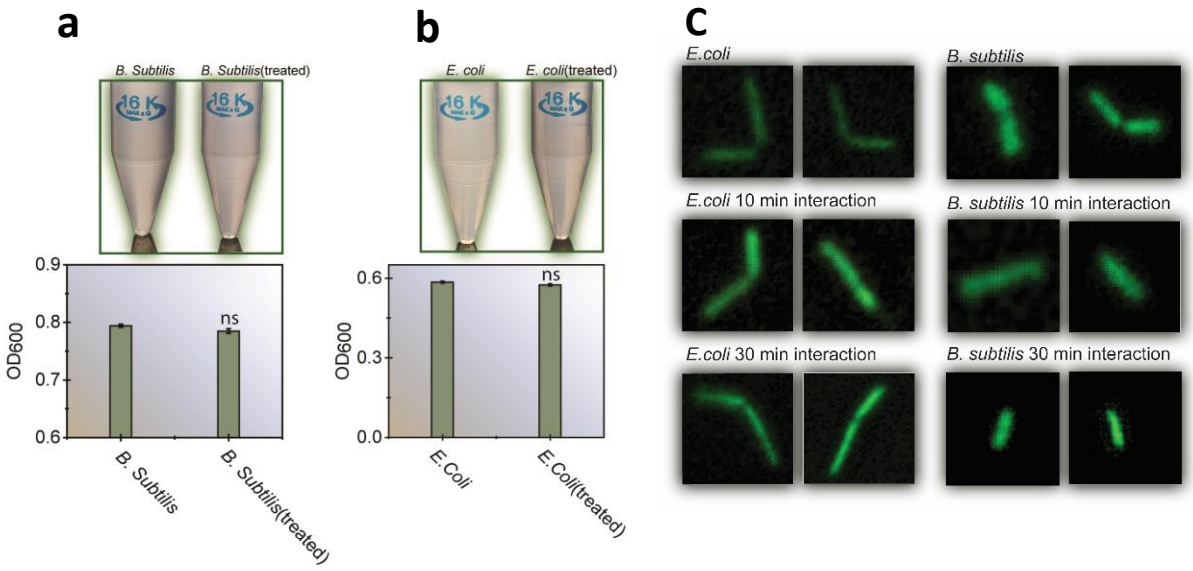

Figure S5| The antibacterial effect of ferricyanide over *B. subtilis* (a) and *E. coli* (b) measured through turbidity measurement. The corresponding visual appearance is shown in the falcon tube. (c) Viability staining of *E. coli* and *B. subtilis* in the presence and absence of 5 mM $K_3Fe[CN]_6$.

Discussion for Figure S5.

The previous reports suggested that $K_3Fe[CN]_6$ is not lethal to bacteria at a concentration of 5 mM, but it is lethal at doses higher than 10 mM[4]. Figure S5 presents the antibacterial activity of $K_3Fe[CN]_6$ with *B. subtilis* and *E. coli* when treated for 18 hrs. The incubation of *B. subtilis* and *E. coli* in LB broth with $K_3Fe[CN]_6$ for 18 hrs is shown above. The analysis shows there are no significant changes from the OD_{600} measurements with control. This shows that $K_3Fe[CN]_6$ at concentration 5mM used in our EAST measurements is suitable to observe the cell activity of the bacterium. However, the interaction between the $K_3Fe[CN]_6$ (5 mM) and the bacterium in our method is very short (less than ca. 1.5 min), thus figure S5 a and b showing the growth in presence of 5 mM $K_3Fe[CN]_6$ at 18 h is not a reasonable comparison. So, fluorescent viability staining was performed, AO/EtBr staining of *E. coli* and *B. subtilis* in presence of 5 mM $K_3Fe[CN]_6$ at 0 min, 10 min, and 30 min is presented in figure S5 c, these observations carried out in conditions similar to the EAST for 5mM $K_3Fe[CN]_6$ concludes that *E. coli* and *B. subtilis* response was not interfered by 5 mM $K_3Fe[CN]_6$ during EAST measurements.

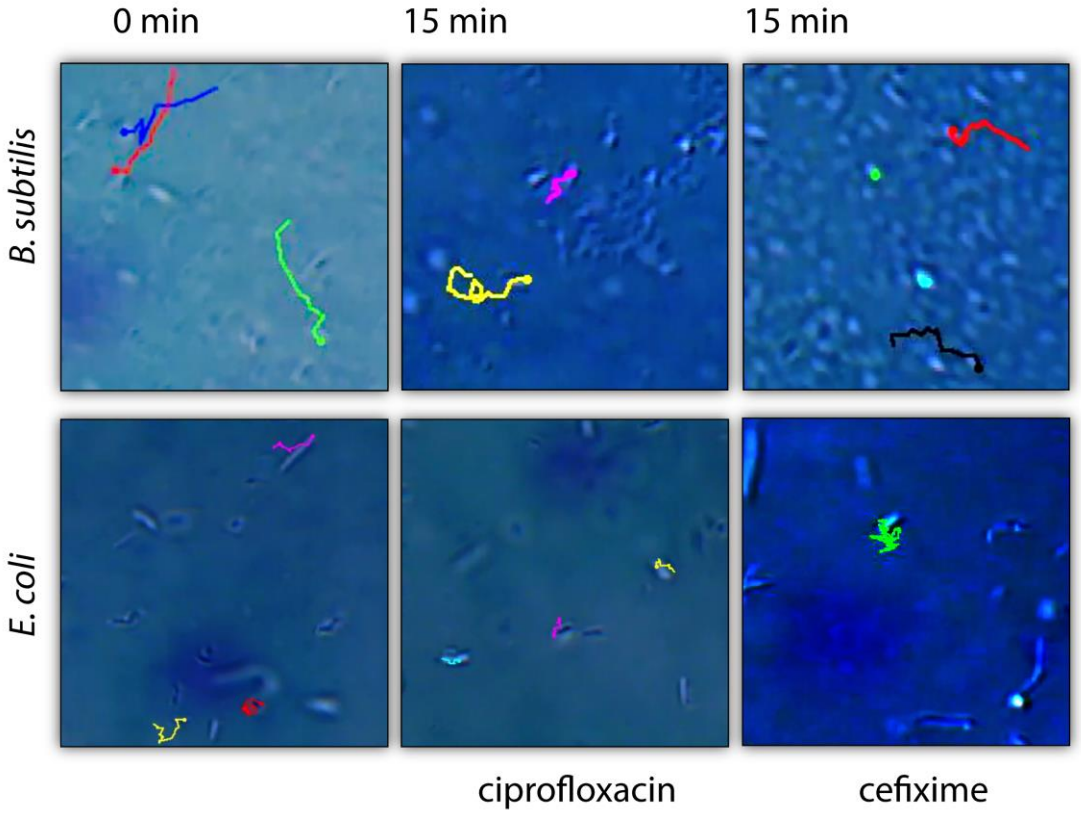

Figure S6| Color inverted time lapse microscopy images of *E. coli* and *B. subtilis*, measured at 0 min and 15 min for ciprofloxacin and cefixime. The trajectory obtained through manual tracking

Sample	Ra (Roughness Average) nm	Rq (Root mean square roughness) nm	Rz (Average Maximum height of the profile) nm
Control	**95.38**	**120.3**	**254.8**
Ciprofloxacin	**64.43**	**76.78**	**173.4**
Cefixime	**59.37**	**80.21**	**150.9**

Table S1| Calculated Roughness average (Ra), Root mean square roughness (Rq), and Average maximum height of the profile (Rz) values of Cefixime and Ciprofloxacin of two distinct mechanisms of action.

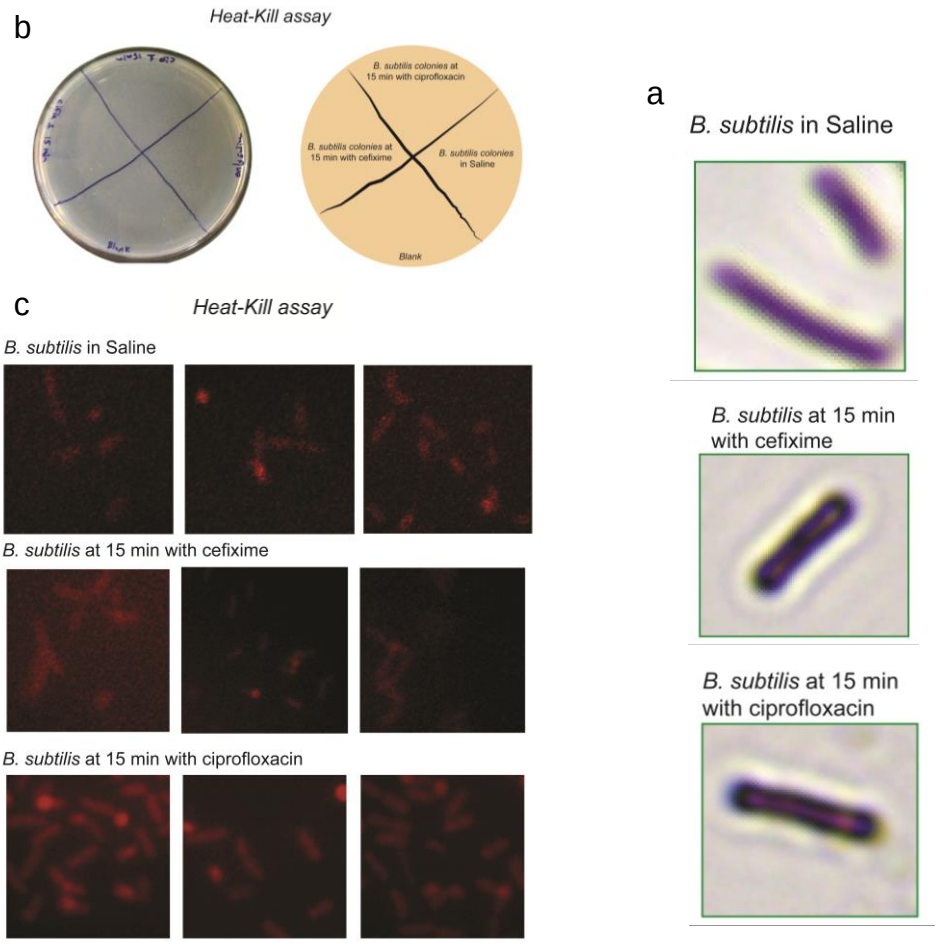

Figure S7| a, The appearance of safranin stain (in Schaeffer-Fulton method) suggests that there is no formation of endospore at different steps of interaction. **b,** Heat-killed *B. subtilis* cells plated on nutrient-rich LB plates. **c,** Further confirmation of Heat-kill assay with EtBr/AO fluorescent stain, which shows the presence of only dead vegetative-cells of *B. subtilis* at different stages of interaction.

Discussion for Figure S7.

The gram-positive *B. subtilis* at unfavorable conditions tend to form endospores. The trigger for sporulation is not only nutritional, but it involves a complex process which is discussed in detail elsewhere[5]. Figure S7 shows the results of heat-killed assay and optical/ fluorescent microscopy images of *B. subtilis*. For analysis, we looked at the possible steps in our method where endospore could form, i.e., during the transfer of *B. subtilis* to saline where the nutrient is deficient. The obtained results show that *B. subtilis* in the presence of cefixime and ciprofloxacin as well as in absence in saline showed no signs of endospore formation. This is further proved

using fluorescent microscopy. This is due to the culture condition used in our study. The Luria Bertani medium as a nutrient-rich medium was used which produced no spores, which is also reported in the previous works[3]. For the formation of endospores use of Difco sporulation medium, nutrient deficient and ion rich medium is required[6,7]. None of which were employed in our work. conclusively, we took the vegetative cells for EAST due to the above-mentioned reasons we did not observe any endospore during EAST.

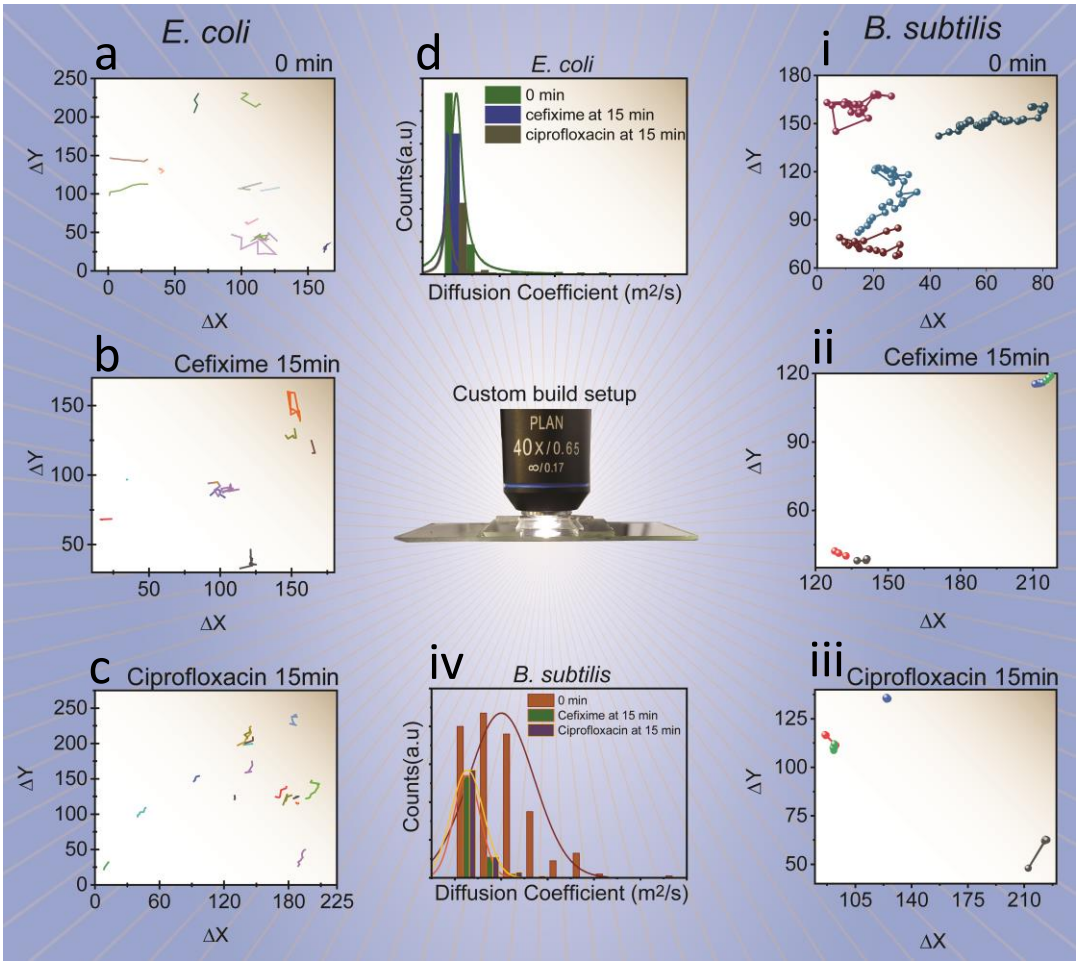

Figure S8| a, b, and **c** show the tracked trajectory of *E. coli* in the presence of ciprofloxacin and cefixime at different time points, their corresponding diffusion coefficient is plotted in **d** with the distribution. **i, ii,** and **iii** plots the tracked trajectory of *B. subtilis* in the presence of ciprofloxacin and cefixime at different time points, their corresponding diffusion coefficient is plotted in **iv** with the distribution.

Discussion for Figure S8.

As the manual measurement made by a human could be prone to error or bias, we used the automated mosaic particle tracker package(Fiji) to further validate the results of rapid EAST within 15 min. Figure S8 plots the trajectory of *E. coli* and *B. subtilis* with the diffusion coefficient, decrease in diffusion coefficient and trajectory additional confirms the rapid EAST in support of Figure S6.

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