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Subminimal inhibitory concentrations of ampicillin and mechanical stimuli cooperatively promote cell-to-cell plasmid transformation in *Escherichia coli*

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ABSTRACT

Horizontal gene transfer (HGT) is a bacterial evolution tool for improved survival. Although several environmental stimuli induce or promote HGT, the diversity and complexity of the environmental factors have not been sufficiently elucidated. In this study, we showed that the biofilm culture of *Escherichia coli* at the air–solid interface in the presence of a subminimal inhibitory concentration (sub-MIC) of ampicillin (~0.5–4 μg/mL) and subsequent mechanical stimulation (rolling small glass balls, ϕ = 5–8 mm) cooperatively promoted horizontal plasmid transfer without the usual competence-inducing conditions. Either of the two treatments promoted plasmid transfer at a lower frequency than when the treatments were combined. The effect of several parameters on the two treatments was tested and then optimized, achieving a high frequency of plasmid transfer (up to 10^{-6} per cell) under optimal conditions. Plasmid transfer was DNase-sensitive for both treatments, demonstrating its mechanism of transformation. Plasmid transfer occurred using various *E. coli* strains, plasmids, ball materials, shaking conditions, and even the mechanical stimulation of brushing the biofilm with a toothbrush, indicating the conditional flexibility of this phenomenon. This is the first demonstration of the promoting effect of the combination of a sub-MIC antibiotic and mechanical stimulation on horizontal plasmid transfer between *E. coli* cells via transformation. Regarding environmental bacterial physiology, the aggregations or biofilms of bacterial cells may encounter both sub-MIC antibiotics and mechanical stimuli in some specific environments, therefore, this type of HGT could also occur naturally.

1. Introduction

Horizontal gene transfer (HGT) is a bacterial evolution tool that improves survival in a variety of habitats (Bushman, 2002; Thomas and Nielsen, 2005; de la Cruz, 2020). However, HGT in the human environment often causes adverse effects or poses potential threats to public health because of the occurrence of novel pathogenic bacteria and antibiotic-resistant bacteria (Bushman, 2002; Morabito, 2014; Lerminiaux and Cameron, 2019; Mochizuki and Maeda, 2020).

E. coli can develop modest natural competence under several environmental conditions (Baur et al., 1996; Bauer et al., 1999; Tsen et al., 2002; Woegerbauer et al., 2002; Maeda et al., 2003, 2004; Sun et al., 2006; Matsumoto et al., 2016a; Komiyama and Maeda., 2020; Riva et al., 2020). Transformation in *E. coli* can be also artificially induced by several stimuli, including $CaCl_2$, heat shock, polyethylene glycol, and electrical shock (Green and Sambrook, 2012). A subminimal inhibitory concentration (sub-MIC) of antibiotics has been proposed to influence several aspects of bacterial cell physiology besides inhibiting bacterial cell growth (Andersson and Hughes, 2014). Certain antibiotics have been reported to induce transformation with added plasmid DNA in naturally transformable bacteria, *Streptococcus pneumoniae* (Prudhomme et al., 2006) and *Legionella pneumophila* (Charpentier et al., 2011). Wu et al. (2020) reported that a sub-MIC of antibiotics facilitated artificial transformation in chemically-competent *E. coli* cells with purified plasmid DNA. The mechanical penetration of mineral nanoneedles [e.g., chrysotile (Yoshida et al., 2001) and sepiolite (Wilharm et al., 2010; Green and Sambrook, 2012)] into the cell's surface in the presence of plasmid DNA has been also reported to induce transformation of *E. coli* and a few other bacteria, termed the Yoshida effect (Yoshida et al., 2001). Some of more macro-level mechanical stimuli, such as shaking with glass beads (Benov and Al-Ibraheem, 2002), flow and/or stretch on rigid surface (Sunuwar et al., 2020; Sirisaengtaksin et al., 2020), etc., have been also reported to affect membrane integrity and cell physiology in *E. coli* cells, although their effects on HGT are still unknown.

We recently revealed that horizontal plasmid transfer by transformation occurs between *E. coli* cells in coculture under conditions that are feasible in the environment (Maeda et al., 2006; Ando et al., 2009;

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Abbreviations

HGT	horizontal gene transfer
AS	air–solid
CTC-PT	cell-to-cell plasmid transformation
TSB	tryptic soy broth

Etchuuya et al., 2011; Matsumoto et al., 2016b; Sugiura et al., 2017; Hashimoto et al., 2019). We named this phenomenon cell-to-cell plasmid transformation (CTC-PT) (Etchuuya et al., 2011). CTC-PT is considered to result from coincidental DNA release from cells and DNA uptake by neighboring cells (Etchuuya et al., 2011; Sugiura et al., 2017; Hasegawa et al., 2018; Hashimoto et al., 2019). Therefore, CTC-PT tends to favor the conditions of high cell density, such as in an air–solid (AS) biofilm (Maeda et al., 2006; Ando et al., 2009; Hashimoto et al., 2019) formed on the interface between solid media and air. In *E. coli*, CTC-PT occurs spontaneously at a low frequency (Maeda et al., 2006; Ando et al., 2009; Etchuuya et al., 2011); however, it can be significantly promoted in the presence of certain environmental factors, such as high temperatures (Hashimoto et al., 2019) and phages (Sugiura et al., 2017). Thus, we presume that there may be other environmental factors that can facilitate CTC-PT.

This study aimed to investigate the CTC-PT-promotion effects of the addition of sub-MIC ampicillin (amp) to *E. coli* cultures and mild mechanical stimulation by rolling small glass balls on *E. coli* AS biofilms. Several parameters were experimentally tested and optimized, and the transformation mechanism was demonstrated. The possible significance as an environmental phenomenon is also discussed.

2. Materials and methods

2.1. E. coli strains and materials

The *E. coli* strains and plasmids used in this study were previously described (Etchuuya et al., 2011; Matsumoto et al., 2016b; Hashimoto et al., 2019). Strains MG1655 (*F*$^-$, *λ*$^-$, *rph-1*), W3110 (*F*$^-$, *λ*$^-$, *IN (rrnD-rrnE)1, rph-1*), DH5 (*F*$^-$, *deoR, recA1, endA1, hsdR17(rK*$^-$*, mK*$^+$*), supE44, l*$^-$*, thi-1, gyrA96, relA1*), BW25113 (*F*$^-$, *rrnB, ΔlacZ4787, HsdR514, Δ(araBAD)567, Δ(rhaBAD)568, rph-1*), and BW25113 *ΔaraC::kan*^{r} (a mutant strain of BW25113 in the Keio collection, *ΔaraC::kan*^{r} on the chromosome) as well as the nonconjugative plasmids pHSG299 (a kanamycin [kan]-resistant cloning vector with a pMB1 origin of replication), pSY510 (a chloramphenicol [cam]-resistant, cloning vector with a p15A origin of replication), and pGBM1 (a streptomycin [str]-resistant medium-copy cloning vector containing the mutated pSC101 replicon) were obtained from the National BioResource Project *E. coli* Strain (National Institute of Genetics, Japan). Plasmid pHSG299-cam (*cam*^{r}) was a pHSG299 derivative that we previously constructed to replace *kan*^{r} with *cam*^{r}. Plasmid pER24y-8ksm (accession no. AB905284) was a nonconjugative, str-resistant natural plasmid that we previously isolated from ECOR24 (Ochman and Selander, 1984), a natural *E. coli* strain. Amp, cam, and Luria–Bertani (LB) powder (Lennox) were obtained from Sigma (St. Louis, MO, USA), tryptic soy broth (TSB) from Becton Dickinson (Franklin Lakes, NJ, USA), phosphate-buffered saline (PBS) tablets from Takara Bio (Kyoto, Japan), and distilled water (DNase- and RNase-free, molecular biology grade) from Invitrogen (Carlsbad, CA, USA). Biodyne A nylon membrane filters (pore size: 0.45 μm) were obtained from Pall (Port Washington, NY, USA) and DNase I (bovine pancreas, grade II) from Roche (Basel, Switzerland). Twelve-well microplates (flat bottom, tissue-culture treated) and small balls of various materials were obtained from AS ONE Corp. (Osaka, Japan), including soda-lime glass balls (ϕ = 1, 3, 5, and 8 mm), alumina balls (ϕ = 3, 5, and 8 mm), polypropylene and nylon balls (ϕ = 5/16 inches [7.9 mm]), and stainless steel balls (ϕ = 1/4 inches [6.4 mm]). Toothbrushes [Clinica Advantage, bristle material: polybutylene terephthalate (https://www.plastics-material.com/pbt/), bristle hardness: normal] were obtained from Lion Corporation (Tokyo, Japan). Agar powder (guaranteed reagent grade), kan, str, and other general reagents were obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan).

2.2. E. coli culture in the presence or absence of sub-MIC amp

An AS biofilm culture of *E. coli* cells was performed as previously described (Maeda et al., 2006; Hashimoto et al., 2019), except that culture was performed in 12-well culture plates in the presence or absence of sub-MIC amp. The combination of DH5, pHSG299, and pSY510 was used as the standard system, unless special conditions are mentioned. Typically, two populations of the same strain harboring either pHSG299 or pSY510 were precultured separately in LB broth containing suitable antibiotics (kan: 75 μg/mL or cam: 100 μg/mL) at 37 °C for 6 h with shaking. The two strains were then suspended in fresh PBS, their turbidities were measured at an optical density of 600 nm (OD_{600}) to estimate the number of cells, and they were mixed at a 1:1 ratio (4×10^9 cells/mL each). For the AS biofilm culture, 20-μL aliquots (8×10^7 cells) were then spotted onto sterilized nylon membrane filter pieces (13 mm in diameter) placed on TSB agar (1.5% w/v) containing various concentrations of amp in the wells of 12-well culture plates and cultured at 37 °C for 16 h. For the experiment shown in Fig. 1-C, AS biofilm coculture was performed with BW25113 *ΔaraC::kan*^{r} and DH5 harboring any of pHSG299-cam, pSY510, pGBM1, or pER24y-8ksm, and two antibiotics (kan and either of cam or str) were used for transformant (BW25113 *ΔaraC::kan*^{r} harboring any of these plasmids) selection. A liquid culture experiment (Fig. 1-E) was performed under similar conditions, except that the two strains were cocultured in 600 μL liquid TSB medium containing sub-MIC amp with shaking at 600 rpm, and a lower concentration of amp (1 μg/mL for DH5) was used because of the higher sensitivity of liquid-cultured cells to amp.

2.3. Mechanical stimuli using glass balls and others on horizontal plasmid transfer in E. coli as biofilm

We used glass balls as mechanical stimuli to determine their effect on horizontal plasmid transfer in *E. coli* AS biofilm. The glass balls were washed once in detergent, rinsed three times in pure water, and sterilized by autoclaving. For the standard protocol, three sterilized glass balls (ϕ = 8 mm) were put onto AS biofilm formed on one of the wells of a 12-well culture plate. This plate was shaken at 1200 rpm on an MBR-022 microplate shaker (TAITEC, Tokyo, Japan) at 25 °C for 60 s. The cells were recovered with PBS, washed once with PBS, and resuspended in fresh LB broth, and a sample was obtained to measure the turbidity (OD_{600}) to estimate the number of cells. The remaining cells were gently spread onto LB agar plates containing two antibiotics (kan: 75 μg/mL for pHSG299, cam: 100 μg/mL for pSY510 and pHSG299-cam, or str: 50 μg/mL for pGBM1 and pER24y-8ksm) to select transformants that had acquired additional antibiotic resistance. Mechanical stimulation using balls of other materials was also performed using the same method. When the hand-forced rolling of ϕ = 1 mm glass balls were performed in the experiment of Fig. 3-A, a piece of sterilized nylon membrane filter (13 mm in diameter) were placed on the glass balls, and the filter was reciprocally shaken (1 return/s) with a pair of sterilized tweezers put on the filter. In the experiment shown in Fig. 1-C, a step of gene expression for 60 min at 37 °C using SOC medium (Green and Sambrook, 2012) was added between cell recovery after mechanical stimulation and cell plating on LB agar to direct a sufficient expression of antibiotic-resistant genes from the newly introduced plasmids. In the experiment shown in Fig. 1-E, liquid-cultured cells (approximately 1.0×10^9 cells/500 μL) were put onto the nylon membrane placed on TSB agar in the wells of a 12-well microplate and then stimulated with glass balls, as described

above. The plasmid transfer frequency was calculated as the ratio of the transformant number to the total cell number, which was deduced from the OD_{600} value of the cell suspension of each sample just prior to plating. Data in the Figures are presented as means and SD (*t-test, $P < 0.05$, $n = 3$–50; ** t-test, $P < 0.01$, $n = 3$–50; *** t-test, $P < 0.005$, $n = 3$–50). Plasmids were isolated and analyzed according to the conventional protocols (Green and Sambrook, 2012) as previously described (Hashimoto et al., 2019).

For mechanical stimulation using a toothbrush, the AS biofilm on the nylon membrane filter was gently brushed with a sterilized toothbrush by hand shaking for 30 s. The cells attached to the bristles of the toothbrush and cells in a well were recovered by rinsing them with fresh PBS and subsequent centrifugation of the cell suspension. The recovered cells were plated for transformant selection, as described above.

2.4. DNase sensitivity test for plasmid transfer

DNase sensitivity tests were performed by adding DNase I (1.8 μg/30 μL) onto the AS biofilm at 6 or at 15 h after starting the culture. The other protocol was the same as that of other plasmid transfer experiments.

3. Results

3.1. Sub-MIC amp and mechanical stimulation with glass balls individually and cooperatively promote CTC-PT in E. coli

We performed the pretests of CTC-PT under various conditions using sub-MIC amp and mechanical stimulation with glass balls. The resulting typical successful data are shown in Fig. 1-A. The results indicated that these two stimuli individually and cooperatively promoted CTC-PT in *E. coli*. In brief, two DH5 strains independently harboring pHSG299 (*kan*^{r}) or pSY510 (*cam*^{r}) were cocultured in the presence or absence of sub-MIC amp (2 μg/mL) as an AS biofilm on a nylon membrane placed on TSB agar in a well of a 12-well plate, as detailed in Section 2.2. In this experimental system, the two plasmids were compatible with each other, so the horizontal transfer of either one was expected to produce one kind of transformant (*kan*^{r} and *cam*^{r}) harboring both plasmids. Consequently, the amp-treated AS biofilm culture resulted in a 1000-fold increase in transformation frequency compared with the equivalent culture in the absence of amp. When the mechanical stimulus, which was rolling (1200 rpm) three glass balls ($\phi = 8$ mm) for a short time (60 s), was solely applied to the amp-free AS biofilm, an approximately 400-fold increase in CTC-PT was observed. Moreover, the successive treatments of sub-MIC amp during culture and mechanical stimulation after culture resulted in an approximately 50,000-fold increase in CTC-PT compared with no treatment, indicating their cooperative effect. This promotion effect was also observed using four different *E. coli* strains (Fig. 1-B) and using four different plasmids with different replication origins, including a nonconjugative natural plasmid (Fig. 1-C). Plasmid isolation from the transformants confirmed the horizontal transfer of full-size plasmids (Fig. 1-D). Of note, CTC-PT frequency significantly decreased in sub-MIC amp-treated liquid culture compared with the equivalent AS biofilm culture (Fig. 1-E). Likewise, when mechanical stimulation was subsequently applied to the amp-treated liquid culture suspension itself ($\sim 1.0 \times 10^9$ cells/500 μL/well; liquid-layer thickness ≈ 1.0–1.5 mm), the CTC-PT frequency was significantly lower than that of the AS biofilm ($\sim 1.0 \times 10^9$ cells/well; biofilm-layer thickness ≈ 0.5–0.8 mm) (Fig. 1-E). These results indicate the importance of biofilm formation in CTC-PT. Based on these results, we further examined various conditions that could affect this cooperative effect. Hereafter, we call this cooperative-promoting effect caused by the double treatments “co-promotion”.

3.2. Effect of amp concentrations on the co-promotion of CTC-PT

We investigated the dose effect of sub-MIC amp on co-promotion (Fig. 2). Cell growth gradually increased at sub-MICs (<5.0 μg/mL) and almost became equivalent to that in the absence of amp when <1.5 μg/mL. Significant co-promotion was observed over a wide range (0.5–4.0 μg/mL), with a gently-sloping peak at 2.25 μg/mL. As the promoting effect was observed even when amp was <1.5 μg/mL, which unaffected cell growth, it was suggested that this promoting effect involved a mechanism that did not depend on cell growth arrest.

3.3. Effects of various conditions of mechanical stimulus with glass balls or by other methods on the co-promotion of CTC-PT

We then examined the effects of various conditions of mechanical stimulus with glass balls on the co-promotion of CTC-PT (Fig. 3A–D, F). The ball sizes tested were 1, 3, 5, and 8 mm in diameter (Fig. 3-A). The number (180, 21, 8, and 3, respectively) of glass balls per well was adjusted so that their total projected area occupied approximately 40% of the base area of a well. Shaking with 5- and 8-mm balls resulted in the significant promotion of CTC-PT and was the highest with 8-mm balls. Visual observation revealed that the 1-mm balls did not roll at all and that the 8-mm balls rolled best among the sizes tested. The hand-forced rolling of 1-mm balls resulted in significant CTC-PT promotion. Therefore, we considered that the degree of ball rolling is a critical determinant of the promoting effect. Next, shaking at 600–2000 rpm resulted in the significant promotion of CTC-PT (Fig. 3-B). Shaking time for only 5 s resulted in the significant promotion of CTC-PT, and shaking between 30 s and 10 min produced a constant high-level frequency of transformation (Fig. 3-C). Shaking with different apparatus or by hand (Fig. 3-D), shaking using other ball materials (polypropylene, nylon, alumina, and stainless steel) (Fig. 3-E), and shaking without the nylon membrane beneath the biofilm (Fig. 3-F) also resulted in significant CTC-PT promotion. Among other mechanical stimuli besides ball rolling, the brushing of biofilm cells with a toothbrush significantly promoted CTC-PT (Fig. 3-G). Collectively, our results showed that the co-promotion of CTC-PT was permissive with various conditions of mechanical stimuli, indicating conditional flexibility of this phenomenon. Our results also suggested that the direct pressing or rubbing of cell aggregates between two solid materials is critical for CTC-PT promotion.

3.4. Demonstration of transformation mechanism in this experimental system

Based on our results above, we hypothesized that CTC-PT promotion by the double treatments occurs in two steps: 1) plasmid DNA leakage and uptake in biofilm cultures due to the action of sub-MIC amp on the cells and 2) plasmid DNA leakage and uptake induced by the mechanical stimulation of the cells dosed with sub-MIC amp. Since transformation requires extracellular naked DNA, we investigated the sensitivity of CTC-PT to DNase (Fig. 3-H). When DNase was added to the cells at 6 h of amp-treated culture but no mechanical stimulation was performed, the CTC-PT frequency significantly decreased (by 98%) compared with the DNase-free control. This result corresponded to step 1 of our hypothesis. When DNase was added to the cells at 15 h of amp-treated culture and then mechanical stimulus was performed, the CTC-PT frequency was also significantly decreased (by 90%) compared with the DNase-free control. This result corresponds to step 2 of our hypothesis. Collectively, these findings indicate that both steps 1 and 2 are DNase-sensitive, thereby confirming that the transformation mechanism is the major mechanism involved in both steps.

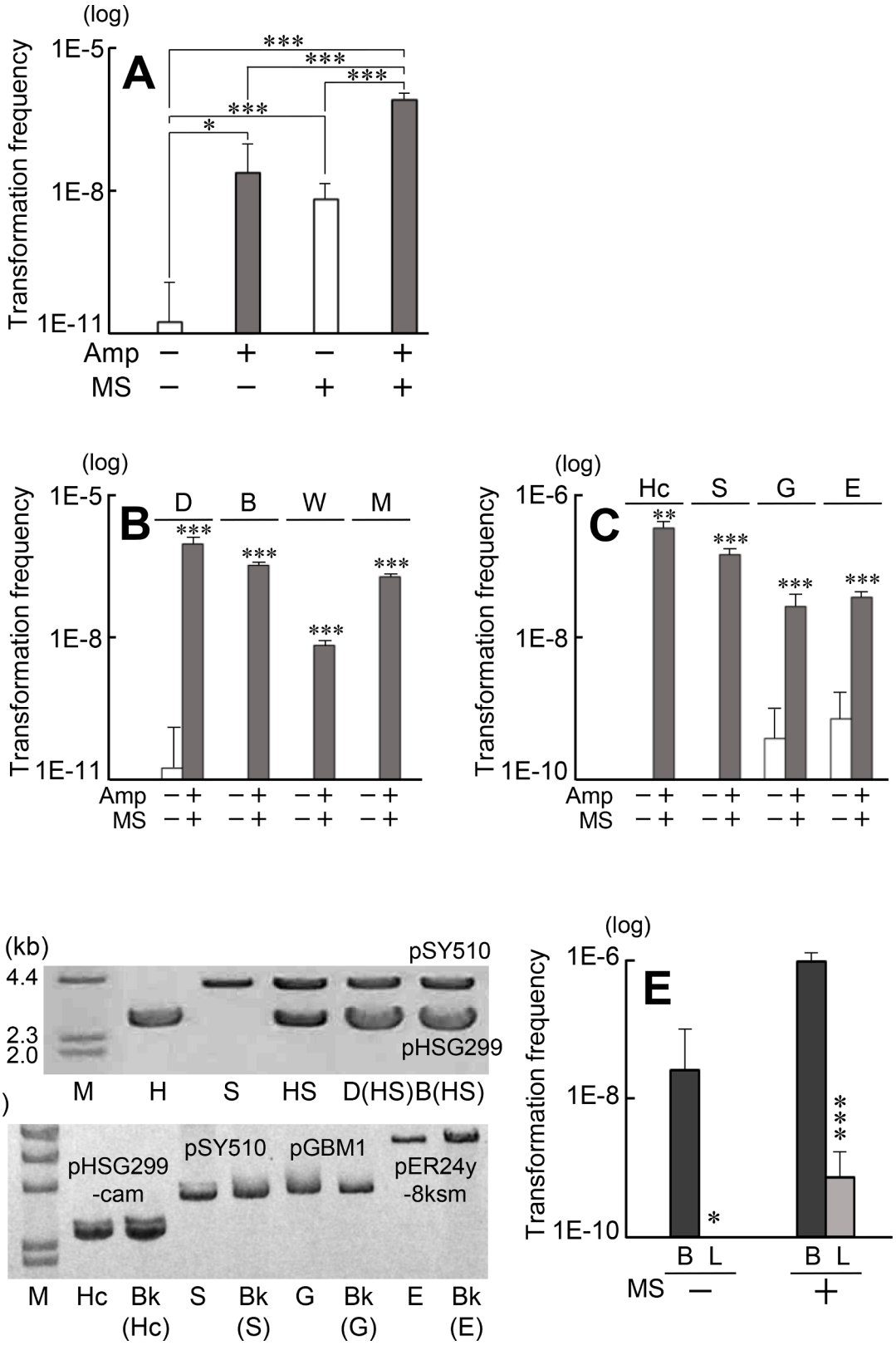

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Fig. 1. (Panel A) Sub-MIC amp and the mechanical stimulus of rolling three glass balls individually and cooperatively promote CTC-PT in *E. coli*. Two DH5 strains independently harboring pHSG299 or pSY510 were cocultured in the presence (+) or absence (−) of sub-MIC amp (2 or 0 μg/mL, respectively) as an AS biofilm in the wells of a 12-well plate. Then, the mechanical stimulus (MS; − or +) of rolling three glass balls (ø = 8 mm) for 60 s by shaking (1200 rpm) was applied to the AS biofilm, as detailed in Section 2.2 and 2.3. (Panel B) Comparison of the effects of sub-MIC amp (Amp: − or +) and mechanical stimulus (MS: − or +) on four *E. coli* strains: DH5 (D), BW25113 (B), W3110 (W), and MG1655 (M). Each *E. coli* strain was cultured as a coculture that independently harbored pHSG299 or pSY510 and then stimulated with glass balls. Amp concentrations suitable for each strain (D: 2, B: 3, W: 1, and M: 4 μg/mL) were adjusted based on the pretest data. (Panel C) Comparison of four plasmids: pHSG299-cam with a pMB1 origin of replication (Hc), pSY510 with a p15A origin of replication (S), pGBM1 with a pSC101 origin of replication (G), and a nonconjugative natural plasmid, pER24y-8ksm (E). The plasmid donor was strain DH5 harboring any of the four plasmids, and the plasmid recipient was BW25113 *ΔaraC::kan*^{r}. They were cocultured with or without sub-MIC amp (Amp: − or +), subsequently mechanically stimulated or not (MS: − or +), and then plated onto an agar medium for the selection of plasmid-acquired BW25113 *ΔaraC::kan*^{r}. (Panel D) Agarose gel (0.8%) electrophoresis of the plasmids isolated from the transformants and cut using *Bam*HI for all plasmids, except pER24y-8ksm where HindIII was used. Lane M, size marker; H, pHSG299 (2676 bp); S, pSY510 (3940 bp); HS, plasmids isolated from control DH5 cells that were artificially transformed with both plasmids; D(HS), plasmids isolated from a DH5 transformant produced in a CTC-PT experiment; B(HS), plasmids isolated from a BW25113 transformant produced in a CTC-PT experiment; Hc, pHSG299-cam (2631 bp); G, pGBM1 (4028 bp); and E, pER24y-8ksm (8531 bp). Lanes Bk(Hc), Bk(S), Bk(G), and Bk(E) are plasmids isolated from BW25113 *ΔaraC::kan*^{r} (Bk) transformants, with each plasmid produced in CTC-PT experiments. (Panel E) Comparison between the data of AS biofilm of strain DH5 (bars B) cultured with amp 2 μg/mL and those of liquid culture (bars L) cultured with amp 1 μg/mL treated with or without mechanical stimulus (MS: + or −). Liquid culture and mechanical stimulation of these cells were conducted as described in Section 2.2 and 2.3. Y-axes of all bar graphs (A, B, C, and E) are shown in log scale.

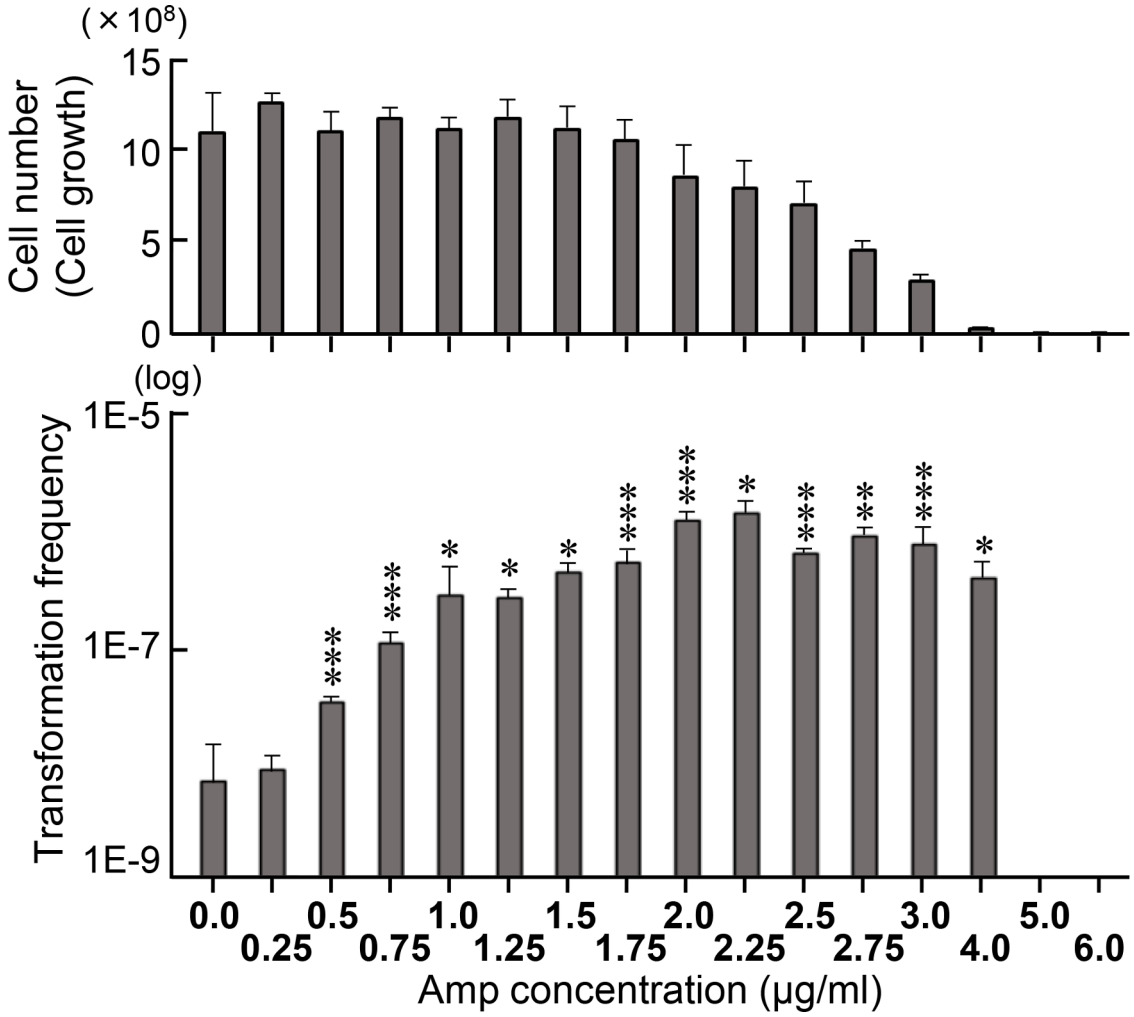
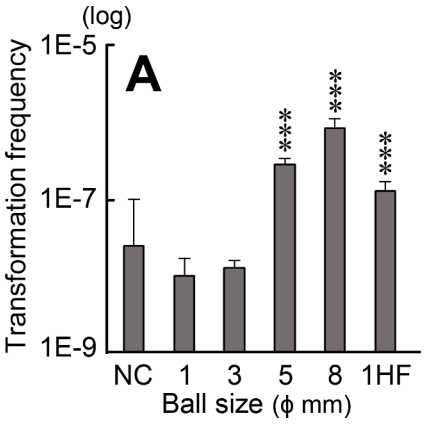
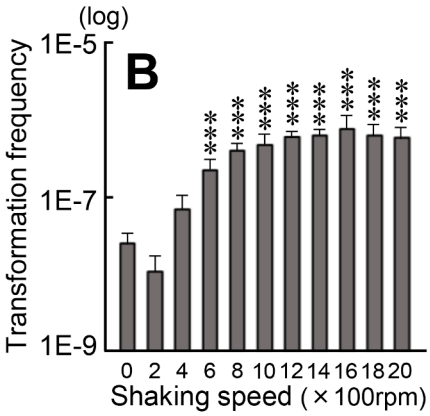
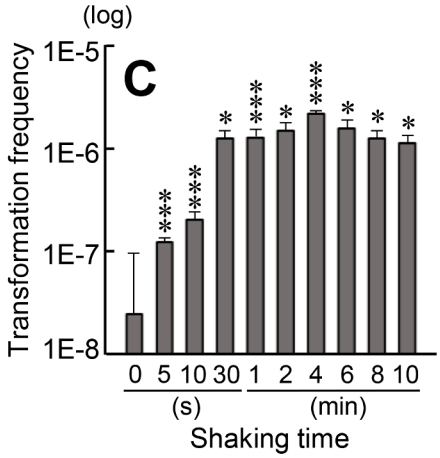
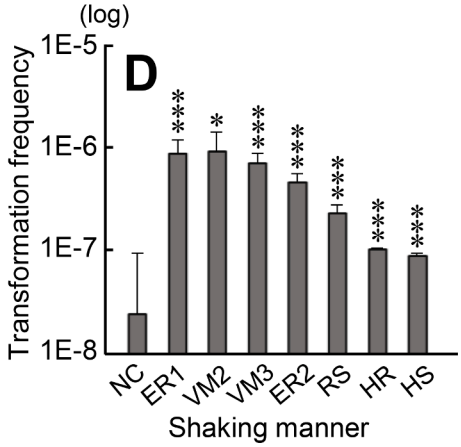


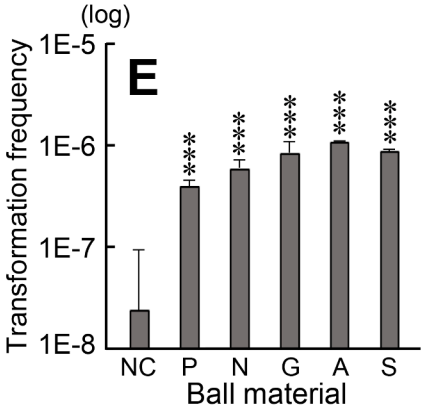
Fig. 2. Effect of amp concentrations on the co-promotion of CTC-PT. The upper graph indicates MIC (5.0 μg/mL) and sub-MIC region (0.25–4.0 μg/mL) in this experiment. X-axis of the upper graph is identical to that of the lower graph. The lower graph shows the effects of various concentrations of amp on CTC-PT. Y-axis of the lower graph is shown in log scale.

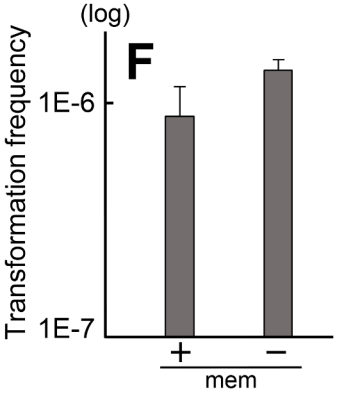


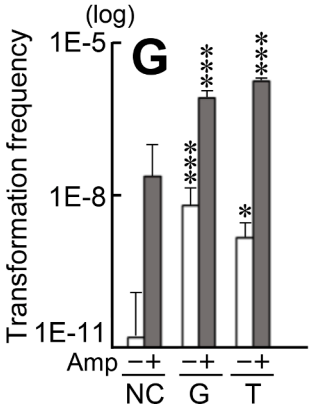












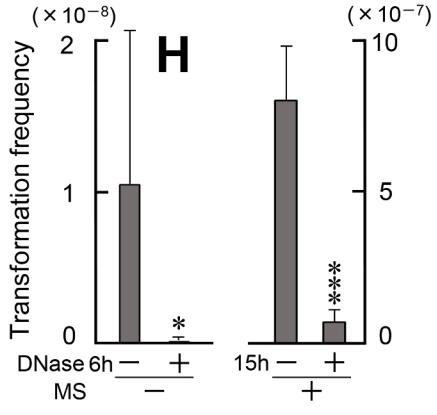


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Fig. 3. Effects of the various conditions of mechanical stimulus on the co-promotion of CTC-PT: (Panel A) ball size (NC: no mechanical stimulus, 1HF: hand-forced rolling of 1-mm balls), (Panel B) shaking speed, (Panel C) shaking time, and (Panel D) Shaking with different apparatus or by hand. The abbreviations of shaking manners are as follows: NC, no mechanical stimulus; ER1, eccentric rotation with an MBR-022 microplate shaker (TAITEC) at 1200 rpm; VM2 and VM3, eccentric rotation with a Vortex Genie2 mixer (Scientific Industries) with a microplate adaptor at setting 2 (approximately 640 rpm) and 3 (approximately 960 rpm); ER2, eccentric rotation with an SHI-2000 shaking incubator (AGC Techno Glass) at 200 rpm; RS, reciprocal shaking with an NTS-1300 Uni Thermo Shaker (EYELA) at 160 rpm; HR, rotation shaking by hand (1 cycle/s); HS, seesaw shaking by hand (1 return/s). (Panel E) Effects of other ball materials (polypropylene: P, nylon: N, glass: G, alumina: A [$\phi = 8$ mm $\times$ 3 balls, respectively], and stainless steel: S [$\phi = 6.4$ mm $\times$ 3 balls]). NC: no mechanical stimulus. (Panel F) Effect of nylon membrane (mem; presence: +, absence: −) between AS biofilm and agar medium by the application of mechanical stimuli with glass balls. (Panel G) Effect of another mechanical stimuli (glass ball: G, toothbrush: T). NC: no mechanical stimulus. (Panel H) DNase sensitivity of CTC-PT. Y-axes of all graphs except panel H are shown in log scale.

4. Discussion

This study demonstrated, for the first time, the cooperative-promoting effect of the combination of sub-MIC amp and mechanical stimuli with glass balls on horizontal plasmid transfer by transformation between *E. coli* cells. Regarding the effect of sub-MIC antibiotics alone, Wu et al. (2020) reported the promoting effect of sub-MIC antibiotics on *E. coli* transformation, but this did not include amp. Furthermore, their study was conducted under artificial conditions using chemically-induced competent cells and purified plasmids. Our study is noteworthy for its use of cells cultured without any other popular competence-inducing treatments and for demonstrating CTC-PT without adding purified plasmids. This situation is closer to natural conditions that are feasible in the environment. Environmental bacterial cells can encounter sub-MIC antibiotics from environmental actinomycetes (Waksman and Woodruff, 1941; Barka et al., 2016) or from antibiotic use in farms (Lander et al., 2012; World Health Organization, 2014a; ter Kuile et al., 2016; Lima et al., 2020; Zalewska et al., 2021; Koch et al., 2021; Mann et al., 2021) and medical situations (Laxminarayan et al., 2013; World Health Organization, 2014b; Zhang et al., 2020). An exemplary and feasible example of both sub-MIC antibiotics and mechanical stimuli occurs in the peristaltic internal organs of antibiotic-fed farm animals or in the feces excreted and stamped on by these animals. Alternatively, based on the results obtained using a toothbrush (Fig. 3-G), brushing biofilms exposed to diluted antibiotics [e.g., brushing dental plaque (Marsh, 2004) in antibiotic-dosed humans or brushing biofilms on the surfaces of sinks and toilet bowls in antibiotic-consuming environments like hospitals (Lindsay and von Holy, 2006)] may provide mechanical stimuli in the presence of sub-MICs to bacterial cells in biofilms. Considering the conditional flexibility of this phenomenon (Figs. 2 and 3), CTC-PT between bacterial cells induced by both stimuli could occur in certain environmental settings outside the laboratory.

Regarding the mechanism of CTC-PT by a co-promotion effect of sub-MIC amp and mechanical stimuli in this study, these two stimuli can individually and cooperatively disturb the cell surface structures of *E. coli* cells. These changes loosen the sealed structures of cell surface membranes, subsequently resulting in the promotion of intercellular plasmid mobilization. Regarding mechanical stress promoting *E. coli* transformation, the Yoshida effect (Yoshida et al., 2001), which occurs due to the mechanical penetration of mineral nano-needles into cell membranes, is known (Wilharm et al., 2010; Green and Sambrook, 2012). Recently, gold or silver nanoparticles have also been reported to promote *E. coli* transformation (Kumari et al., 2017; Nagamani et al., 2019). However, these two effects are unlikely to be involved in the phenomenon presented in our current study because balls and a toothbrush, which showed transformation-promoting effects in our study, are composed of various materials and are not known to possess such specialized nanoscale structures on their surfaces. We hypothesize that not material-specific surface structures but rather some types of simple physical force (pressure, tension, friction, or their combination) on the cells could be involved in this effect. Of note, a CTC-PT promotion was also observed when applying another physical stimulus, high temperature (Hashimoto et al., 2019). In addition to the above effects supposed, other effects, such as increased cell-to-cell interaction by mixing cell aggregate, etc. may also contribute to the promotion of CTC-PT.

Further investigations will be required to elucidate some unsolved points on the mechanism and the universality of co-promotion of CTC-PT, such as the effects of various experimental factors (other antibiotics, other physical or biochemical factors, the ratio of donor and recipient cells, etc.), the molecular-level sequences and the participating molecules driving plasmid DNA release and uptake, the effects on other bacteria, etc. Some of these investigations are underway.

In conclusion, this study demonstrated, for the first time, the cooperative-promoting effect of the combination of sub-MIC amp and mechanical stimuli on horizontal plasmid transfer by transformation between *E. coli* cells. Considering its permissive inducing conditions and relatively high frequency, this phenomenon may naturally occur. Further studies of this phenomenon hold promise for possible developments in bacterial physiology on HGT.

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Declaration of Competing Interest

There are no conflicts of interest to declare.

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References

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12, 465–478. https://doi.org/10.1038/nrmicro3270.

Ando, T., Itakura, S., Uchii, K., Sobue, R., Maeda, S., 2009. Horizontal transfer of non-conjugative plasmid in colony biofilm of *Escherichia coli* on food-based media. World J. Microbiol. Biotechnol. 25, 1865–1869. https://doi.org/10.1007/s11274-009-0070-y.

Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Meier-Kolthoff, J. P., Klenk, H.P., Clément, C., Ouhdouch, Y., van Wezel, G.P., 2016. Taxonomy, physiology, and natural products of Actinobacteria. Microbiol. Mol. Biol. Rev. 80, 1–43. https://doi.org/10.1128/MMBR.00019-15.

Bauer, F., Hertel, C., Hammes, W.P., 1999. Transformation of *Escherichia coli* in foodstuffs. Syst. Appl. Microbiol. 22, 161–168. https://doi.org/10.1016/S0723-2020(99)80061-7.

Baur, B., Hanselmann, K., Schlimme, W., Jenni, B., 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. Appl. Environ. Microbiol. 62, 3673–3678. https://doi.org/10.1128/aem.62.10.3673-3678.1996.

Benov, L., Al-Ibraheem, J., 2002. Disrupting *Escherichia coli*: a comparison of methods. J. Biochem. Mol. Biol. 35, 428–431. https://doi.org/10.5483/bmbrep.2002.35.4.428.

Bushman, F., 2002. Lateral DNA Transfer. Cold Spring Harbor Laboratory Press, New York.

Charpentier, X., Kay, E., Schneider, D., Shuman, H.A., 2011. Antibiotics and UV radiation induce competence for natural transformation in *Legionella pneumophila*. J. Bacteriol. 193, 1114–1121. https://doi.org/10.1128/JB.01146-10.

de la Cruz, F., 2020. Horizontal Gene Transfer: Methods and Protocols. Humana Press, New York.

Etchuuya, R., Ito, M., Kitano, S., Shigi, F., Sobue, R., Maeda, S., 2011. Cell-to-cell transformation in *Escherichia coli*: a novel type of natural transformation involving cell-derived DNA and a putative promoting pheromone. PLoS ONE 6, e16355. https://doi.org/10.1371/journal.pone.0016355.

Green, M.R., Sambrook, J., 2012. Molecular Cloning: a Laboratory Manual, 4th ed. Cold Spring Harbor Laboratory Press, New York.

Hasegawa, H., Suzuki, E., Maeda, S., 2018. Horizontal plasmid transfer by transformation in *Escherichia coli*: environmental factors and possible mechanisms. Front. Microbiol. 9, 2365. https://doi.org/10.3389/fmicb.2018.02365.

Hashimoto, M., Hasegawa, H., Maeda, S., 2019. High temperatures promote cell-to-cell plasmid transformation in *Escherichia coli*. Biochem. Biophys. Res. Commun. 515, 196–200. https://doi.org/10.1016/j.bbrc.2019.05.134.

Koch, N., Islam, N.F., Sonowal, S., Prasad, R., Sarma, H., 2021. Environmental antibiotics and resistance genes as emerging contaminants: methods of detection and bioremediation. Curr. Res. Microb. Sci. 2, 100027 https://doi.org/10.1016/j.crmicr.2021.100027.

Komiyama, Y., Maeda, S., 2020. Natural Transformation in *Escherichia coli*. In: de la Cruz, F. (Ed.), Horizontal Gene Transfer: Methods and Protocols. Humana Press, New York, pp. 179–187. https://doi.org/10.1007/978-1-4939-9877-7_13.

Kumari, M., Pandey, S., Mishra, A., Nautiyal, C.S., 2017. Finding a facile way for the bacterial DNA transformation by biosynthesized gold nanoparticles. FEMS. Microbiol. Lett. 364 https://doi.org/10.1093/femsle/fnx081 fnx081.

Lander, T.F., Cohen, B., Wittum, T.E., Larson, E.L., 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep 127, 4–22. https://doi.org/10.1177/003335491212700103.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K.M., Wertheim, H.F.L., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O., 2013. Antibiotic resistance-the need for global solutions. Lancet Infect. Dis. 13, 1057–1098. https://doi.org/10.1016/S1473-3099(13)70318-9.

Lerminiaux, N.A., Cameron, A.D.S., 2019. Horizontal transfer of antibiotic resistance genes in clinical environments. Can. J. Microbiol. 65, 34–44. https://doi.org/10.1139/cjm-2018-0275.

Lima, T., Domingues, S., Da Silva, G.J., 2020. Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. Vet. Sci. 7, 110. https://doi.org/10.3390/vetsci7030110.

Lindsay, D., von Holy, A., 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. J. Hosp. Infect. 64, 313–325. https://doi.org/10.1016/j.jhin.2006.06.028.

Maeda, S., Kakihara, N., Koishi, Y., 2003. Competency development of *Escherichia coli* in foodstuffs. Microbes Environ 18, 100–103. https://doi.org/10.1264/jsme2.18.100.

Maeda, S., Sawamura, A., Matsuda, A., 2004. Transformation of colonial *Escherichia coli* on solid media. FEMS Microbiol. Lett. 236, 61–64. https://doi.org/10.1016/j.femsle.2004.05.023.

Maeda, S., Ito, M., Ando, T., Ishimoto, Y., Fujisawa, Y., Takahashi, H., Matsuda, A., Sawamura, A., Kato, S., 2006. Horizontal transfer of nonconjugative plasmids in a colony biofilm of *Escherichia coli*. FEMS Microbiol. Lett. 255, 115–120. https://doi.org/10.1111/j.1574-6968.2005.00072.x.

Mann, A., Nehra, K., Rana, J.S., Dahiya, T., 2021. Antibiotic resistance in agriculture: perspectives on upcoming strategies to overcome upsurge in resistance. Curr. Res. Microb. Sci. 2, 100030 https://doi.org/10.1016/j.crmicr.2021.100030.

Marsh, P.D., 2004. Dental plaque as a microbial biofilm. Caries Res. 38, 204–211. https://doi.org/10.1159/000077756.

Matsumoto, A., Sekoguchi, A., Murakami, Y., Imai, J., Kondo, K., Shibata, Y., Maeda, S., 2016a. Competence development and horizontal plasmid transfer in natural *Escherichia coli* strain. In: Mendez-Vilas, A. (Ed.), Microbes in the Spotlight: Recent Progress in the Understanding of Beneficial and Harmful Microorganisms. BrownWalker Press, Boca Raton, pp. 468–473.

Matsumoto, A., Sekoguchi, A., Imai, J., Kondo, K., Shibata, Y., Maeda, S., 2016b. Natural *Escherichia coli* strains undergo cell-to-cell plasmid transformation. Biochem. Biophys. Res. Commun. 481, 59–62. https://doi.org/10.1016/j.bbrc.2016.11.018.

Mochizuki, M., Maeda, S., 2020. First and broad detection of three typical carbapenemase genes on the surfaces of commercially available spices worldwide and isolation of complete NDM-1 genes from black pepper, cumin, and clove. bioRxiv [Preprint]. https://doi.org/10.1101/2020.06.09.141614.

Morabito, S., 2014. Pathogenic *Escherichia coli*: Molecular and Cellular Microbiology. Caister Academic Press, Norfolk.

Nagamani, G., Alex, S., Soni, K.B., Anith, K.N., Viji, M.M., Kiran, A.G., 2019. A novel approach for increasing transformation efficiency in *E. coli* DH5α cells using silver nanoparticles. 3 Biotech 9, 113. https://doi.org/10.1007/s13205-019-1640-9.

Ochman, H., Selander, R.K., 1984. Standard reference strains of *Escherichia coli* from natural populations. J. Bacteriol. 157, 690–693, 0.1128/jb.157.2.690-693.1984.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., Claverys, J.P., 2006. Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae 313, 89–92. https://doi.org/10.1126/science.1127912.

Riva, F., Riva, V., Eckert, E.M., Colinas, N., Cesare, A.D., Borin, S., Mapelli, F., Crotti, E., 2020. An environmental *Escherichia coli* strain is naturally competent to acquire exogenous DNA. Front. Microbiol. 11, 574301 https://doi.org/10.3389/fmicb.2020.574301.

Sirisaengtaksin, N., Odem, M.A., Bosserman, R.E., Flores, E.M., Krachler, A.M., 2020. The *E. coli* transcription factor GrlA is regulated by subcellular compartmentalization and activated in response to mechanical stimuli. Proc. Natl. Acad. Sci. USA. 117, 9519–9528. https://doi.org/10.1073/pnas.1917500117.

Sugiura, C., Miyaue, S., Shibata, Y., Matsumoto, A., Maeda, S., 2017. Bacteriophage P1*vir*-induced cell-to-cell plasmid transformation in *Escherichia coli*. AIMS Microbiol 3, 784–797. https://doi.org/10.3934/microbiol.2017.4.784.

Sun, D., Zhang, Y., Mei, Y., Jiang, H., Xie, Z., Liu, H., Chen, X., Shen, P., 2006. *Escherichia coli* is naturally transformable in a novel transformation system. FEMS Microbiol. Lett. 265, 249–255. https://doi.org/10.1111/j.1574-6968.2006.00503.x.

Sunuwar, L., Yin, J., Kasendra, M., Karalis, K., Kaper, J., Fleckenstein, J., Donowitz, M., 2020. Mechanical stimuli affect *Escherichia coli* heat- stable enterotoxin-cyclic GMP signaling in a human enteroid Intestine-Chip model. Infect. Immun. 88 https://doi.org/10.1128/IAI.00866-19 e00866-19.

ter Kuile, B.H., Kraupner, N., Brul, S., 2016. The risk of low concentrations of antibiotics in agriculture for resistance in human health care. FEMS Microbiol. Lett. 363, fnw210. https://doi.org/10.1093/femsle/fnw210 fnw.

Thomas, C.M., Nielsen, K.M., 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3, 711–721. https://doi.org/10.1038/nrmicro1234.

Tsen, S.D., Fang, S.S., Chen, M.J., Chien, J.Y., Lee, C.C., Tsen, D.H.L., 2002. Natural plasmid transformation in *Escherichia coli*. J. Biomed. Sci. 9, 246–252. https://doi.org/10.1159/000059425.

Waksman, S.A., Woodruff, H.B., 1941. *Actinomyces antibioticus*, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. J. Bacteriol 42, 231–249. https://doi.org/10.1128/jb.42.2.231-249.1941.

Wilharm, G., Lepka, D., Faber, F., Hofmann, J., Kerrinnes, T., Skiebe, E., 2010. A simple and rapid method of bacterial transformation. J. Microbiol. Methods. 80, 215–216. https://doi.org/10.1016/j.mimet.2009.12.002.

Woegerbauer, M., Jenni, B., Thalhammer, F., Graninger, W., Burgmann, H., 2002. Natural genetic transformation of clinical isolates of *Escherichia coli* in urine and water. Appl. Environ. Microbiol. 68, 440–443. https://doi.org/10.1128/AEM.68.1.440-443.2002.

World Health Organization., 2014a. WHO guidelines on use of medically important antimicrobials in food-producing animals. https://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-eng.pdf [Accessed June 7, 2019].

World Health Organization., 2014b. Antimicrobial resistance: global report on surveillance. https://apps.who.int/iris/bitstream/handle/10665/112642/9789241564748_eng.pdf [Accessed June 7, 2019].

Wu, H.Y., Shi, D.Y., Yang, D., Yin, J., Yang, Z.W., Li, J.W., Yang, W., Jin, M., 2020. Putative environmental levels of levofloxacin facilitate the dissemination of antibiotic-resistant *Escherichia coli* via plasmid-mediated transformability. Ecotoxicol. Environ. Saf. 195, 110461 https://doi.org/10.1016/j.ecoenv.2020.110461.

Yoshida, N., Ikeda, T., Yoshida, T., Sengoku, T., Ogawa, K., 2001. Chrysotile asbestos fibers mediate transformation of *Escherichia coli* by exogenous plasmid DNA. FEMS Microbiol. Lett. 195, 133–137. https://doi.org/10.1111/j.1574-6968.2001.tb10510.x.

Zalewska, M., Błażejewska, A., Czapko, A., Popowska, M., 2021. Antibiotics and antibiotic resistance genes in animal manure – consequences of its application in agriculture. Front. Microbiol. 12, 610656 https://doi.org/10.3389/fmicb.2021.610656.

Zhang, S., Huang, J., Zhao, Z., Cao, Y., Li, B., 2020. Hospital wastewater as a reservoir for antibiotic resistance genes: a meta-analysis. Front. Public Health 8, 574968. https://doi.org/10.3389/fpubh.2020.574968.