Low-intensity ultrasound promotes the horizontal transfer of resistance genes mediated by plasmids in *E. coli*

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Abstract

Widespread of pathogenic bacteria resistant to antibiotics has become a worldwide public health concern. Conjugative transfer between bacteria is an important mechanism for the horizontal transfer of antibiotic resistance genes. Ultrasound has been widely applied in many fields, but the effect of ultrasound on horizontal transfer of antibiotic-resistant genes is still not clear. We discovered that low-intensity ($\leq 0.05 \text{ W/cm}^2$) ultrasound had no effect on bacterial growth and survival rates, but increased the permeability of cell membrane, and consequentially elevated the transfer rates of plasmid. Low-intensity ultrasound enhanced conjugation between bacteria, induced expression of conjugation genes *TrpBp* and *TrfAp*, and inhibited expression of global regulatory genes *KorA*, *KorB*, *TrbA*, and *TrbK*. In conclusion, low-intensity ultrasound promoted horizontal transfer of antibiotic-resistant genes by enhancing conjugation and regulating expression of horizontal transfer-related genes.

Keywords Low-intensity ultrasound · Horizontal gene transfer · Antibiotic resistance genes · Conjugation · Bacteria

Introduction

The spread and evolution of antibiotic resistance in bacteria has become one of the most serious threats to global public health (Ippolito et al. 2010). Every year over 25,000 patients in Europe, > 23,000 in United States, and > 38,000 in Thailand die from infectious diseases caused by multiresistant bacteria. One of the main causes of the spread of antibiotic-resistant genes is due to the selective pressures led by the excessive use and misuse of antibiotics in medicine and animal feedstuffs (Kummerer 2001), as well as manure application on agricultural fields (Heuer et al. 2011). The selective pressures promote mutation accumulation or horizontal transfer of antibiotic-resistant gene between bacteria to speed up the spread of antibiotic-resistant bacteria (Smets and Barkay 2005), and horizontal gene transfer is considered

as the major one (Davison 1999). Horizontal gene transfer is a process that can compensate for the otherwise clonal mode of prokaryotic life, affecting microbial adaptation, speciation, and evolution (Smets and Barkay 2005). Some modern techniques have been reported to strengthen horizontal gene transfer, like nanomaterials (Qiu et al. 2012). It is urgent to identify those techniques potentiating horizontal gene transfer to control the spread of antibiotic-resistant bacteria.

Ultrasound has been widely used in water treatment and therapy of diseases. For example, ultrasound is used to improve the therapeutic effects in transdermal drug delivery, thrombolysis, and transfection of gene vectors (Hsu et al. 2013; Miller et al. 2017; Sun et al. 2013). It has been reported that low-intensity ultrasound enhances the bactericidal effect of antibiotics towards bacteria including planktonic bacteria, bacterial biofilms, and chlamydia (Yu et al. 2012). The synergistic bactericidal effects of low-intensity ultrasound are mediated by acoustic cavitation effect. Ultrasound is able to elevate membrane permeability due to sonoporation, which in turn increases the permeability of cytomembrane over a relatively short period, resulting in exchange of substances between the interior and exterior of cells. Thus, non-permeant antibiotics are enhanced and delivered into bacteria (Lammertink et al. 2015; Yudina et al. 2011). Plasmid transfer into bacteria

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is also dramatically promoted by low-intensity ultrasound (Song et al. 2007). However, it is unknown whether or how ultrasound affects antibiotic-resistant gene transfer between bacteria. Since ultrasound increases the permeability of bacterial cytomembrane, we hypothesize that ultrasound may promote the horizontal transfer of multidrug-resistance genes between bacteria.

In this study, we systematically observed the effect of low-intensity ultrasound on the horizontal transfer of antibiotic-resistant gene between bacteria. We demonstrate that ultrasound significantly increased conjugation and impacted expression of horizontal gene transfer-related genes. The promotion of ultrasound on horizontal transfer of antibiotic-resistant genes between bacteria is essential for guiding the manufacture and application of ultrasound in environment and clinical treatment, and also in the evaluation of the possible consequences on ecology and bioremediation.

Methods

Ultrasonic irradiation treatment and measurement of bacterial activity

Bacterial suspensions were aliquoted into 6-well plates, and were separately irradiated with ultrasound. Ultrasonic irradiation was performed as previously reported (Dong et al. 2017). After treatment, all bacterial suspensions were further shaken at 37 °C for 24 h. The optical density at 600 nm (OD_{600}) of the bacterial suspension was measured at indicated incubation time points using a microplate reader (ThermoFisher, Waltham, MA, USA). The survival rate of bacteria was determined by a flow cytometer (BD FACS-Vantage SE, San Diego, CA, USA).

Bacterial permeability measurement

The bacterial permeability was measured as previously described (Dong et al. 2017). Briefly, fluorescein diacetate (FDA) was added to 5-ml bacterial suspensions to a final concentration of 100 µg/ml followed by incubation at 37 °C in the dark for 15 min. Then propidium iodide (PI) was added to the suspensions to a final concentration of 60 µg/ml and incubated for 5 min. After staining, the bacterial suspension was subjected to ultrasonic irradiation, and then the bacteria were collected by centrifuge at 5000 rpm for 10 min, and 4 °C. The cell pellet was washed with phosphate buffer saline (PBS) twice to remove the unstained dyes, and the pellet was re-suspended in 1-ml PBS. Flow cytometry was utilized to measure intensity of FDA and PI in the cells.

Conjugation experiment

After ultrasonic irradiation, the conjugation cultures were placed in an incubator to mate. After incubation for an indicated time, the cultures were vigorously mixed and diluted, and appropriate dilutions were spread on Luria–Bertani (LB) agar plates containing rifampicin. After overnight incubation at 37 °C, the colonies were counted and the results were presented as cfu/ml and transfer frequency.

Transmission electron microscope (TEM)

The cell pellet was re-suspended in pre-cooled 2.5% glutaraldehyde fixative, and fixed at 4 °C for 2 h. The cells were collected by centrifugation at 1000g for 20 min, and the cell pellet was washed with 0.2-mM citric acid twice. Then, the samples were fixed with 2% OsO_4 in anhydrous acetone that contained 8% dimethoxypropane for 2 days at 4 °C, followed by dehydration using an ethanol gradient method and treatment with propylene oxide, and then embedded in Epon812 resin. The samples were sectioned into slices about 50-nm thickness with an ultramicrotome (Leica Ultracut-R). The samples were post-stained with 2% aqueous uranyl acetate for 15 min, and Reynold's lead citrate for 5 min. The samples were examined with a Philips CM120 TEM.

mRNA levels determined by real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cells using a RNA extraction kit (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed to cDNA with a reverse transcription kit (Invitrogen, USA). Real-time PCR was carried out using a SYBR Green I (TaKaRa, Otsu, Japan) with Applied Biosystems 7300 real-time PCR system. The primers for genes can be provided as required, and 16SrRNA acts as internal control.

Statistical analysis

Data from this study were expressed as the mean value $\pm$ standard deviation. GraphPad Prism version 6 (GraphPad Software; La Jolla, CA, USA) was used for statistical analysis. Comparisons between different groups were made with Student's *t* test, one-way analysis of variance, or general linear model repeated measures. Statistical significance was defined as $P < 0.05$.

Results

Low-intensity ultrasound does not affect bacterial vitality

To test the sensitivity of bacteria to ultrasound, *E. coli* suspensions were treated with different intensities of ultrasonic irradiation for 5 min. The irradiated bacteria were cultured for 24 h, and the optical density at 600 nm (OD_{600}) of bacteria was measured at time points: 0, 4, 6, 8, 12, and 24 h. As shown in (Fig. 1a), the growth of bacteria was not affected by 0.05 W/cm² ultrasound, but significantly inhibited by the other two higher intensities. The inhibition effect of the 0.1 W/cm² ultrasound on bacterial activity started at the time point of 12 h, while the inhibition effect by the intensity of 0.3 W/cm² occurred as early as 4 h after incubation. After 24 h culture, the survival rate of irradiated *E. coli* was not impacted by the low-intensity of ultrasonic irradiation, but significantly reduced by the other two higher intensities (Fig. 1b). We defined the ultrasound ≤ 0.05 W/cm² intensity as the low-intensity ultrasound. These results demonstrated that the vitality of *E. coli* was not influenced by the low-intensity of ultrasonic irradiation.

Low-intensity ultrasonic irradiation elevates bacterial permeability

To investigate if ultrasound treatment changes the permeability of cells, fluorescent intensity test was conducted after *E. coli* was exposed to ultrasonic irradiation. The use of

the cell permeable esterase-substrate fluorescein diacetate (FDA) and the cell impermeant nucleic acid stain propidium iodide (PI) to assess cell permeability was combined. FDA accumulates only in cells containing intact cell membrane. If the cell permeability increases, FDA staining is weakened, and PI staining is increased. As shown in (Fig. 2a), FDA staining was decreased by 50% comparing to control samples in response to all ultrasound intensities for 5 min. Consistently, ultrasound treatment dramatically increased PI staining dependent on the intensity of ultrasonic irradiation (Fig. 2b), suggesting the bacterial permeability increased after ultrasound treatment. These data indicate that bacterial permeability is elevated by ultrasound treatment, even by low-intensity ultrasound (0.05 W/cm²), which does not impact bacterial vitality.

Low-intensity ultrasonic irradiation promotes conjugative transfer of RP4

Since low-intensity ultrasound treatment increased bacterial permeability, we hypothesized that low-intensity ultrasound treatment promotes conjugative transfer of genes between bacteria. To test this hypothesis, *E. coli* HB101, harboring rifampicin resistant gene-contained plasmid RP4, was mixed with *E. coli* K12, and incubated to induce mating between these two types of bacteria. Then, the mixture was irradiated by low-intensity ultrasound at 0, 4, 8, 12, and 24 h after incubation. As shown in (Fig. 3a), ultrasound treatment increased the number of *E. coli* K12 resistant to rifampicin over 100-folds, and this effect depended on mating time (Fig. 3a). Next, we examined the effect of bacterial concentration on the plasmid

Fig. 1 Low-intensity ultrasound does not affect bacterial vitality. *E. coli* suspensions were irradiated by ultrasound at indicated intensity, and then cultured for 24 h **a** The optical density at 600 nm (OD_{600}) was determined with spectrometry at culture time points 0, 4, 8, 12, and 24 h. * $P < 0.05$, compared to the control group at the each time

point ($n = 6$ independent experiments). **b** After 24 h culture, the survival rate of irradiated *E. coli* was determined by flow cytometry. The survival rate was calculated as a percentage of the bacterial activity against the control group, * $P < 0.05$, compared to the control group ($n = 6$ independent experiments)

Fig. 2 Low-intensity ultrasonic irradiation elevates bacterial permeability. *E. coli* suspensions were irradiated by ultrasound at different intensities for 5 min, and then subjected to FDA dye staining (**a**)

and PI staining (**b**). The fluorescent intensity was measured by flow cytometry. * $P < 0.05$, compared to control group ($n = 6$ independent experiments)

Fig. 3 Low-intensity ultrasonic irradiation promotes conjugative transfer of plasmids. **a** The effect of mating times on the conjugative transfer of RP4. 10^9 cfu/ml bacteria were treated with 0.05 W/cm^2 ultrasonic irradiation at pH 7.0, 25 °C. * $P < 0.05$, compared to the control group at the each time point ($n = 6$ independent experiments).

b The effect of bacterial concentration on the conjugative transfer of RP4. 10^9 cfu/ml bacteria mating for 8 h were treated with 0.05 W/cm^2 ultrasonic irradiation at pH 7.0, 25 °C. * $P < 0.05$, compared to the control at each concentration ($n = 6$ independent experiments)

transfer. As shown in (Fig. 3b), the higher the bacterial concentration was applied, the more efficient was the RP4 plasmid transferred. These results proved our hypothesis: low-intensity ultrasound promotes horizontal transfer of genes.

Low-intensity-promoted conjugation is confirmed by transmission electron microscopy (TEM)

To confirm the conjugation between bacteria induced by low-intensity ultrasound, we used TEM to observe the

conjugation. In the control group, the bacterial membranes were intact, cell borders were clear, and the cytoplasm was compact and evenly distributed (Fig. 4a). After treatment of low-intensity ultrasound, the cytoplasm of the bacteria agglomerated, and parts of the cell membrane were undefined. Moreover, many spherical, highly dense particles were observed in these bacteria (Fig. 4b). In the control group of mating incubation, fewer compact electron-dense areas were observed between the outer cell membrane of the donor and recipient, although there were many close links

Fig. 4 Low-intensity-promoted conjugation is confirmed by TEM observation. The bacteria in mating incubation were treated or not treated with low-intensity ultrasonic irradiation (0.05 W/cm^2). The morphology of cell membranes was observed in the control (a) and the ultrasound-irradiated (b) bacteria. The conjugative junctions were assessed in the control (c) and the ultrasound-irradiated (d) bacteria. Scale bars, 500 nm

between the bacteria (Fig. 4c). Stimulated by low-intensity ultrasound in mating bacteria, compact electron-dense areas were observed between bacteria, suggesting that conjugation occurred (Fig. 4d). All together, TEM results confirm the conjugation between bacteria promoted by low-intensity ultrasound.

Low-intensity ultrasound regulates expression of conjugation genes and global regulatory genes

Plasmid conjugal transfer is promoted by conjugation genes *TrbBp* and *TrfAp*, and repressed by the major global regulatory genes *KorA*, *KorB*, and *TrbA*, as well as *TrbK*. Low-intensity ultrasonic irradiation strongly stimulated expression of *TrbBp* and *TrfAp* (Fig. 5a), while greatly prevented expression of *KorA*, *KorB*, (Fig. 5b) *TrbA*, and *TrbK* (Fig. 5c). These data indicate that the gene expression manipulated by low-intensity ultrasound is the other mechanism underlying horizontal transfer of antibiotic-resistant genes, in addition to enhancing conjugation.

Discussion

This study investigated the effect of low-intensity ultrasound on horizontal gene transfer between bacteria. Low-intensity ultrasound had no effect on bacterial growth and survival rates, and; however, increased the permeability of cell membrane. Furthermore, the transfer rate of antibiotic gene in plasmid was dramatically promoted by low-intensity ultrasound. Finally, we elucidated two mechanisms underlying low-intensity ultrasound-induced horizontal transfer of genes. One is low-intensity ultrasound enhanced conjugation between bacteria; the other is low-intensity ultrasound-induced expression of conjugation genes *TrbBp* and *TrfAp*, and inhibited global regulatory genes *KorA*, *KorB*, *TrbA*, and *TrbK*.

The increased global documentation shows human pathogenic bacteria, such as *Streptococcus pneumonia* and *Staphylococcus aureus*, developed resistance to multiple classes of antibiotics. This has been identified as one of the key challenges to contemporary infectious-disease control (Smets

Fig. 5 Low-intensity ultrasound regulates expression of conjugation genes and global regulatory genes. 10^9 cfu/mL bacteria mating for 8 h were irradiated with or without 0.05 W/cm² ultrasound. Total RNA was purified and reverse transcribed. Real-time PCR was performed

to determine expression of *TrbBp* and *TrfAp* (a), *KorA* and *KorB* (b), as well as *TrbA* and *TrbK* (c), * $P < 0.05$, compared to control ($n = 6$ independent experiments)

and Barkay 2005), and has become one of the most important clinical challenges (Arias and Murray 2009). Horizontal gene transfer plays a critical role in development of bacteria resistant to multiple classes of antibiotics (Saad et al. 2000). Thus, integrated understanding of horizontal gene transfer and identification of factors associated with enhancing horizontal gene transfer help people control the spread of pathogenic bacteria with antibiotic-resistant genes.

The process of horizontal gene transfer can be divided into at least four steps (Smets and Barkay 2005). First, a gene in the donor organism is ready for transfer. The gene can be packaged into phage particles, or contained in a plasmid, or passive-released to the environment upon cell death. Second, the transfer step takes place dependent or not dependent on physical contact between the donor and recipient organism. Third, the gene enters the recipient organisms. Fourth, the gene is established in the recipient either as a self replicating element or through recombination with, or transposition into, the recipient's chromosome. Clearly, low-intensity ultrasound functions at least at step 2 and 3 to enhance horizontal gene transfer. Ultrasound promoted physical contact between the donor and recipient organism (Fig. 4), and regulated the expression of conjugation genes and global regulatory genes (Fig. 5), to facilitate plasmid transfer.

In this study, RP4 plasmid was used as the vehicle for gene transfer. The RP4 plasmid is a typical IncP α -type plasmid, which is a broad-host-range conjugative plasmid. The conjugative transfer of this IncP plasmid requires the products of the *TrfAp* and *TrbBp* genes. Both *KorA* and *KorB* repress *TrfAp* expression, and *TrbA* and *KorB* severely inhibit the activity of the *TrbBp* promoter (Schreiner et al. 1985; Theophilus et al. 1985). *TrbK* functions in surface exclusion (Lyras et al. 1994). In this study we found that mRNA expression of *KorA*, *KorB*, and *TrbA* was repressed significantly by low-intensity ultrasound (Fig. 5). These results suggest that expression of *TrfA* and *TrbB* may be induced by inhibition of *KorA*, *KorB*, and *TrbA*, and sequentially

enhance the efficiency of transfer (Kostelidou et al. 1999; Zatyka et al. 1997), which may be further promoted by down-regulation of *TrbK*.

Ultrasound is an ideal approach for plasmid or DNA fragment's delivery into cells, including bacteria (Han et al. 2005), fungi (Liu et al. 2006), plants, and mammalian cells (Bao et al. 1997). Compared to high-intensity ultrasound (1–3 MHz) (Han et al. 2005), low-intensity ultrasound (40 kHz) more efficiently promotes plasmid transformation into bacteria (Song et al. 2007). But there is no paper showing the effect of low-intensity ultrasound on horizontal gene transfer between two bacteria, which was reported in this study for the first time.

Conclusion

This study found that low-intensity ultrasound promoted horizontal transfer of antibiotic-resistant genes between bacteria by enhancing conjugation, and regulating the expression of conjugation genes and global regulatory genes. The findings in this study suggest that the application of low-intensity ultrasound should be evaluated carefully so as to not cause public health, environmental, and ecological hazards.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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