

# Ultrasound-mediated DNA transfer for bacteria

Yizhi Song<sup>1,2</sup>, Thomas Hahn<sup>1</sup>, Ian P. Thompson<sup>1,3</sup>, Timothy J. Mason<sup>4</sup>,  
Gail M. Preston<sup>5</sup>, Guanghe Li<sup>2</sup>, Larysa Paniwnyk<sup>4</sup> and Wei E. Huang<sup>1,\*</sup>

<sup>1</sup>Centre for Ecology & Hydrology, Oxford, OX1 3SR, UK, <sup>2</sup>Department of Environmental Science and Engineering, Tsinghua University, Beijing, 100084, People's Republic of China, <sup>3</sup>Begbroke Directorate, University of Oxford Science Park, Yarnton, Oxford, OX5 1PF, <sup>4</sup>Faculty of Health and Life Sciences, Coventry University, Coventry, CV1 5FB and <sup>5</sup>Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, UK

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## ABSTRACT

In environmental microbiology, the most commonly used methods of bacterial DNA transfer are conjugation and electroporation. However, conjugation requires physical contact and cell-pilus-cell interactions; electroporation requires low-ionic strength medium and high voltage. These limitations have hampered broad applications of bacterial DNA delivery. We have employed a standard low frequency 40 kHz ultrasound bath to successfully transfer plasmid pBBR1MCS2 into *Pseudomonas putida* UWC1, *Escherichia coli* DH5 $\alpha$  and *Pseudomonas fluorescens* SBW25 with high efficiency. Under optimal conditions: ultrasound exposure time of 10 s, 50 mM CaCl<sub>2</sub>, temperature of 22°C, plasmid concentration of 0.8 ng/ $\mu$ l, *P. putida* UWC1 cell concentration of  $2.5 \times 10^9$  CFU (colony forming unit)/ml and reaction volume of 500  $\mu$ l, the efficiency of ultrasound DNA delivery (UDD) was  $9.8 \pm 2.3 \times 10^{-6}$  transformants per cell, which was nine times more efficient than conjugation, and even four times greater than electroporation. We have also transferred pBBR1MCS2 into *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25 with efficiencies of  $1.16 \pm 0.13 \times 10^{-6}$  and  $4.33 \pm 0.78 \times 10^{-6}$  transformants per cell, respectively. Low frequency UDD can be readily scaled up, allowing for the application of UDD not only in laboratory conditions but also on an industrial scale.

## INTRODUCTION

Transferring DNA to cells is a fundamental technique of molecular cloning, which has revolutionized molecular biology. In clinical practice, gene therapy and RNAi rescue

is dependent on the success of DNA/RNA delivery (1). However, in environmental microbiology, the vast majority of prokaryotes (>99%) in natural environments are unculturable (2), and therefore not amenable to DNA delivery using traditional culture-dependent DNA delivery methods. Even when prokaryotes can be cultured *in vitro*, genetic manipulation is frequently impeded because of the lack of efficient, non-invasive and simple methods for DNA delivery. Efficient transfer of DNA to natural microbial communities, such as those found in groundwater and soils, could be used to rapidly introduce novel gene functions, and therefore change the structure and functionality of microbial communities. Delivery of regulatory genes or small regulatory RNA (sRNA) could be used to silence bacterial genes and alter bacterial behaviour, and sRNA and regulatory genes have the potential to be used as novel antibiotics against multidrug-resistant bacteria, or to inhibit bacterial biofilm formation (3). Therefore, a simple and efficient DNA/RNA delivery system is essential for the successful application of all of these technologies.

Biological methods for bacterial DNA transfer include conjugation, gene transformation and transduction (4–8). Biological methods such as conjugation and transduction usually require a specific DNA donor or host strain to achieve bacterial DNA transfer (6), while gene transformation is limited to a few naturally competent groups (8). Physical methods for gene transfer include microinjection, particle bombardment, electroporation, laser irradiation and sonoporation (9). Microinjection and particle bombardment are interference techniques: they directly penetrate the cell membrane to introduce nucleic acids into cells (9). Laser irradiation employs a laser to change cell permeability and allows DNA to be transferred (9,10), but requires physical contact of laser and cells. Most of these physical techniques are only applied to small-scale samples of eukaryotic cells. In practice, the most commonly used methods for bacterial gene transfer are

\*To whom correspondence should be addressed. Tel: +44 (0)114 2225796; Fax: +44 (0)114 2225701; Email: weihuang@yahoo.com, w.huang@sheffield.ac.uk

Present address:

Wei E. Huang, Kroto Research Institute, North Campus, University of Sheffield, Broad Lane, Sheffield S3 7HQ, UK

conjugation and electroporation, along with heat shock transfer, which is mostly used with *Escherichia coli* cells. Conjugation requires physical contact of recipient and a donor with conjugative plasmid (11,12), or the participation of the third bacterium with a helper plasmid (13). Electroporation is highly efficient but requires a low-ionic strength medium and a high voltage for operation (14,15). Neither method can be adapted for a broad range of DNA delivery applications.

Ultrasound DNA delivery (UDD) is an ideal approach for plasmid or DNA fragments delivery, which has been intensively studied in the context of eukaryotic transformation and gene therapy recent years (16–21). The mechanism of UDD is primarily based upon a cavitation effect, which physically generates reversible porosity in the cell membrane (22–24). UDD has several advantages that make it an attractive technique for DNA delivery. First, UDD can, in theory, deliver DNA or RNA to any type of cell including bacteria (25), fungi, plants (26) and mammalian cells (16,18,19,21,27,28). Second, UDD does not require ion-free media, and therefore can be applied to cells growing in natural media or human body fluids. Third, UDD is a non-invasive method, which does not require direct physical contact. However, in all previous studies, the ultrasound frequency used for transferring DNA was in the ranges of 1–3 MHz (16–21). Such frequencies have worked well with eukaryotic cells but the efficiency of bacterial transformation at these frequencies is low compared to conventional methods for bacterial transformation (25).

We report here that a standard low frequency (40 kHz) ultrasound clean bath can be used to successfully and efficiently deliver plasmid pBBR1MCS2 into *Pseudomonas putida* UWC1. Optimized conditions gave a delivery efficiency of  $9.8 \pm 2.3 \times 10^{-6}$  transformants per cell, which was significantly higher than the results of conjugation and even electroporation. We have also successfully transferred pBBR1MCS2 to *Escherichia coli* DH5 $\alpha$  and *Pseudomonas fluorescens* SBW25 with  $1.16 \pm 0.13 \times 10^{-6}$  and  $4.33 \pm 0.78 \times 10^{-6}$  transformants per cell. Low frequency UDD has several advantages over high frequency UDD and other transformation methods for industrial and environmental applications. Low frequency UDD can be used to transform bacteria at room temperature and in a wide range of media. Furthermore, ultrasonic apparatus operating in the frequency range of 20–40 kHz can be readily scaled up, allowing for the application of UDD on large scales, for example, introducing DNA to natural microbial communities in order to promote enhanced biodegradation of pollutants in groundwater or wastewater treatment plants.

## MATERIALS AND METHODS

### Chemicals and media

All chemicals were obtained from Sigma-Aldrich Co., UK and were analytical-grade reagents unless otherwise stated. Luria–Bertani (LB) medium was used for the cultivation of bacteria. SOC medium (29) was used to recover antibiotic-resistant cells after electroporation and

UDD. Ampicillin and rifampicin were used at a final concentration of 100  $\mu$ g/ml. Fifty microgram per millilitre of kanamycin was used with *E. coli* DH5 $\alpha$  and *P. putida* UWC1 and 100  $\mu$ g/ml with *P. fluorescens* SBW25. LB agar containing 100  $\mu$ g/ml rifampicin (LBR) was used to obtain total cell counts of *P. putida* UWC1. LB agar with 50 and 100  $\mu$ g/ml kanamycin (LBK) was used to select for transformants of *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25. LBK supplemented with 100  $\mu$ g/ml rifampicin (LBKR) was used to identify and count *P. putida* UWC1 transformants carrying pBBR1MCS2.

### Bacteria and plasmids

*E. coli* DH5 $\alpha$ , *P. putida* UWC1 and *P. fluorescens* SBW25 were used as recipients of plasmid. *P. putida* UWC1 is a spontaneous rifampicin-resistant mutant of *P. putida* KT2440 (30). The broad-host-range cloning vector pBBR1MCS2 (5144 bp) (31,32), hosted in *E. coli* JM109 (pBBR1MCS2) was employed as delivery DNA. Plasmid DNA was extracted and purified from overnight cultures of *E. coli* JM109, DH5 $\alpha$ , *P. putida* UWC1 or *P. fluorescens* SBW25 using a QIAprep Spin Miniprep kit (QIAGEN, UK). DNA concentration was determined using a GeneQuant Pro RNA/DNA calculator (Amersham Pharmacia Biotech, USA).

### Ultrasound apparatus

Two different ultrasound systems were used with a frequency of either 40 or 850 kHz. The 40 kHz ultrasound apparatus was a standard ultrasonic cleaning bath 375H (Langford Electronics Ltd., Coventry, UK) with a maximal electrical energy output of 75 W. The ultrasound water bath dimension was 240  $\times$  135  $\times$  100 (mm, L  $\times$  W  $\times$  H). The higher frequency ultrasound apparatus was an 850 kHz ultrasonic bath (Meinhart Ultraschalltechnik, Germany, K80-5) connected to a transducer. The K80-5 generator could generate ultrasound at four intensities, with a maximal electrical energy output of 140 W.

Ultrasound may have a heating effect, so temperature was monitored throughout the experiments using a Digital Test Thermometer (Brannan Thermometers, UK).

To determine the ultrasound power in the plasmid transfer system, the temperature ( $T$ ) was recorded against time ( $t$ ), at 15 s intervals, over a period of 4 min using a thermocouple placed in the reaction itself. From the  $T$  versus  $t$  data, the temperature rise at zero time ( $dT/dt$ ), can be estimated by either constructing a tangent to the curve at time = 0, or by curve fitting the data to a polynomial in  $t$ . The ultrasonic power actually entering the system can be obtained from this by substituting the value of ( $dT/dt$ ), at time zero (obtained from either method) into the following equation (33):

$$\text{Power} = \frac{dT_c M}{dt} \quad 1$$

where  $c_p$  is heat capacity of the solvent (J/kg/K) and  $M$  the mass of solvent used (kg).

Power measurements were achieved using the 1.75-ml flat-bottom glass vial (Richardsons of Leicester Ltd., UK).

### Ultrasound plasmid transfer

All experiments were conducted with 5–24 replicates and means  $\pm$  SE are presented. Statistical tests were performed using Excel (Microsoft, Inc.). Prior to transformation, single colonies of *P. putida* UWC1, *E. coli* DH5 $\alpha$  or *P. fluorescens* SBW25 were inoculated and incubated in 10 ml LB broth for 13–15 h at 28°C, washed three times in 0.85% NaCl and then resuspended in 10-ml transformation medium. One, three or five hundred microlitre solutions of *P. putida* UWC1, 500  $\mu$ l of *E. coli* DH5 $\alpha$  or *P. fluorescens* SBW25 cells and plasmid pBBR1MCS2 were mixed and transferred to 1.75-ml flat-bottom glass vials (Richardsons of Leicester Ltd., UK). The vials were placed in the centre of the 375H ultrasound bath. The vials were fully immersed in the ultrasound water bath, which contained 1.6l of tap water, and exposed to 40 kHz ultrasound. Unless otherwise stated, cell concentrations were 10<sup>8–9</sup> CFU (colony forming unit)/ml, the final concentration of plasmid applied to UDD was 0.8 ng/ $\mu$ l cells, ultrasound exposure time was 10 s and UDD was carried out at 21.5–22.9°C throughout the experiments. After ultrasound treatment, 900  $\mu$ l SOC medium were added to 100  $\mu$ l reaction mixture. Cells were incubated in a 28°C incubator with 150 r.p.m. shaking for 2 h. Cells were diluted and plated onto appropriate media to obtain cell counts.

To attempt plasmid transfer to *P. putida* UWC1 at 850 kHz K80-5, vials containing 100  $\mu$ l reaction mixture (10<sup>8</sup> CFU/ml and 0.8 ng/ $\mu$ l of plasmid pBBR1MCS2) were placed at the centre of the ultrasound bath. The exposure time of UDD was 5–30 s for each intensity of constant ultrasound, and 30 s for ultrasound pulse. Temperature was controlled and maintained at 20°C by cycling cooling water.

*The effect of UDD on plasmid integrity.* To examine the effect of ultrasound on plasmids, vials containing 80 ng of pBBR1MCS2 DNA in 100  $\mu$ l of 100 mM CaCl<sub>2</sub> solution were separately exposed to 40 kHz ultrasound for 0, 5, 10, 30, 30 and 60 s. After ultrasound treatment, each sample was transferred to a 1.5-ml Eppendorf tube and added to 10  $\mu$ l 3 M sodium acetate, 200  $\mu$ l absolute ethanol and 1  $\mu$ l glycogen. Samples were incubated on ice for 30 min and then centrifuged at 163 000g for 10 min. The supernatant was removed and the DNA pellet was dried in an Eppendorf Concentrator 5301 (Hamburg, Germany) for 10 min. The DNA pellet was then resuspended in 10  $\mu$ l deionized water. The recovered plasmid DNA was examined by agarose-ethidium bromide gel electrophoresis.

*Medium effect on UDD efficiencies.* To assess medium effect on UDD, three times saline washed *P. putida* UWC1 cells were mixed with plasmid DNA and resuspended in four different types of media: LB medium, phosphate buffer solution (PBS), 100 mM MgSO<sub>4</sub> and 100 mM CaCl<sub>2</sub>.

Since CaCl<sub>2</sub> was found to enhance ultrasound plasmid transfer, it was then further investigated by performing

UDD in CaCl<sub>2</sub> with final concentrations of 0, 1, 10, 50, 100, 200, 300 or 500 mM.

*Temperature effect on UDD efficiencies.* To determine the temperature effect, UDD was performed at 0, 22, 28 and 42°C.

*Plasmid concentration and UDD efficiencies.* UDD was carried out with plasmid DNA at final concentrations of 0, 0.08, 0.2, 0.5, 0.8, 1, 2, 5 or 8 ng/ $\mu$ l cells.

*Cells concentration and UDD efficiencies.* UDD was carried out at cell concentrations of 10<sup>7</sup>, 10<sup>8</sup> and 10<sup>9</sup> CFU/ml.

*UDD of E. coli DH5 $\alpha$  and P. fluorescens SBW25.* The UDD conditions for *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25 were: 10 s exposure time, 50 mM CaCl<sub>2</sub>, 22°C, 1 ng/ $\mu$ l plasmid DNA of pBBR1MCS2 and cell concentrations of *E. coli* DH5 $\alpha$  5.8  $\times$  10<sup>8</sup> CFU/ml and *P. fluorescens* SBW25 1.1  $\times$  10<sup>9</sup> CFU/ml. The total reaction volume was 500  $\mu$ l.

### Conjugation and electroporation

Electroporation and conjugation were used as positive controls for plasmid transformation of *P. putida* UWC1. For electroporation, an overnight culture of *P. putida* UWC1 was washed three times and resuspended in 10% glycerol. Electroporation used similar conditions to the optimized conditions reported by Cho *et al.* (34). Fifty microlitres of cell suspension and 1  $\mu$ l plasmid (27 ng/ $\mu$ l) were mixed in 0.2-cm electroporation cuvettes and electroporated under conditions of 12.5 kV/cm and 25  $\mu$ FD using a Bio-rad gene pulser (Bio-Rad Laboratories, UK). Cells were mixed with 950  $\mu$ l of SOC immediately after electroporation and cells were incubated for 2 h at 28°C. For conjugation, 50  $\mu$ l of an overnight culture of *P. putida* UWC1 (10<sup>9</sup> cells/ml) and 50  $\mu$ l of an overnight culture of *E. coli* JM109 (10<sup>9</sup> cells/ml), harbouring pBBR1MCS2, were mixed by vortexing for 10 s and loaded onto an 0.2- $\mu$ m GTBP membrane filter (Millipore, UK) and then placed on a LB agar plate. Cell were incubated for 16 h incubation at 28°C, and then washed from the filters and recovered. After electroporation or conjugation, cells were plated on LBR and LBKR plates to obtain total cell and transformant counts.

### DNA manipulation and sequencing

Ultrasound transformants of *P. putida* UWC1, *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25 (pBBR1MCS2) were verified by plasmid digestion, plasmid sequencing and 16S DNA sequencing of recipient cells. Ten transformants of each species were randomly selected from different batches of LBK plates and streaked on fresh LBK plates. Single colonies were picked from each fresh LBK plate and inoculated into LBK liquid medium. Plasmid DNA extracted from *P. putida* UWC1, *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25 (pBBR1MCS2) and from original host *E. coli* JM109 was digested with NotI endonuclease (New England Biolabs, UK), and analysed using a 1%

agarose-ethidium bromide gel. For further confirmation, plasmids were sequenced using primer BBR1\_for (5'-CCG AAG CCC AAC CTT TCA TAG AA-3').

16S DNA of *P. putida* UWC1 transformants was amplified by polymerase chain reaction (PCR) using cells from a single colony as a DNA template. The universal 16S primers for PCR were forward 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse 530r (5'-GTA TTA CCG CGG CTG CTG-3'). PCR amplifications were performed with initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final additional 72°C for 10 min extension. PCR products were isolated from a 1% agarose gel and purified according to the manufacturer's instructions using a QIAquick gel extraction kit (Qiagen Co., UK). 16S PCR products were sequenced using primer 63f.

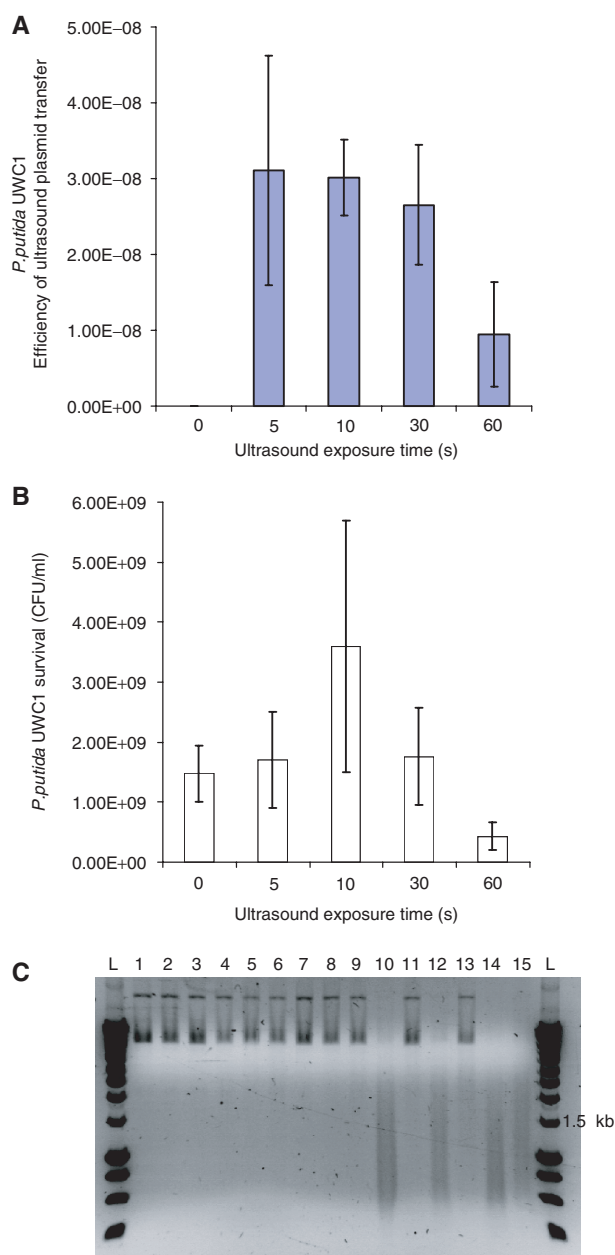
### Nucleotide sequencing and sequence analysis

All DNA samples (PCR products or plasmids) were sequenced using dye terminator sequencing on an Applied Biosystems 3730 DNA analyzer, according to the manufacturer's instructions. DNA sequence analysis was carried out using Blastn for confirmation of sequence homology and sequence data were aligned and edited using BioEdit to confirm correct insertions (Tom Hall, Department of Microbiology, North Carolina State University).

## RESULTS AND DISCUSSION

### Low frequency ultrasound treatment promotes DNA delivery into bacterial cells

Although high frequency (>200 kHz) ultrasound has been reported to promote DNA transfer into mammalian cells (9,16–21), we found that high frequency 850 kHz ultrasound failed to deliver plasmid pBBR1MCS2 into *P. putida* UWC1 (data not shown). However, low frequency 40 kHz ultrasound did promote the transfer of plasmid pBBR1MCS2 into *P. putida* UWC1 (Figure 1A). We observed no transfer of plasmid DNA into *P. putida* UWC1 in the absence of exposure to ultrasound (Figure 1A), which excludes the possibility that transformation was due to natural competence. In all UDD experiments, we used flat-bottom glass vials. Plastic vessels can absorb ultrasound energy and round and smooth bottom vials reflect ultrasound, making the efficiency of UDD low (data not shown). As the ultrasound generator was set at the bottom of the 375H ultrasonic cleaning bath, the alignment of glass vials and generator is critical. We tried nine different positions and found that the efficiency of UDD at the centre point of the 375H water bath was higher than other points. Therefore, all UDD experiments were carried out by positioning a single vial at the centre point of the 375H bath. The ultrasound power at the centre point of the 375H bath was calculated using Equation (1), as described in materials and methods, to be 240 mW/cm<sup>2</sup> in each vial.



**Figure 1.** Ultrasound (40 kHz) exposure time affects the efficiency of plasmid delivery (A), cell survival (B) and plasmid integrity (C). (A) Ultrasound treatment for 5–60 s results in plasmid transfer to *P. putida* UWC1 ( $P < 0.05$ ). Exposure time of 10 s gives the highest transfer efficiency. No plasmid transfer occurred in the absence of ultrasound treatment. (B) Ultrasound reduced *P. putida* UWC1 survival following 60 s exposure time, but shorter treatments had little effect on bacterial survival ( $P < 0.05$ ). (C) Plasmid DNA was prone to cleavage following ultrasound exposure times longer than 30 s: L, ladder (Bioline, UK, HyperLadder I); lanes 1–3, plasmid without ultrasound; lanes 4–6, plasmid treated by ultrasound for 5 s; lanes 7–9, 10 s; lanes 10–12, 30 s and lane 13–15, 60 s.

### Ultrasound exposure time affects cell viability, plasmid integrity and UDD

The damage to cells which is generally associated with ultrasonic energy was first recorded in 1929 in a study of *Bacillus fisheri* in sea water (35). High power ultrasound is

capable of killing bacteria and for many years sonication has been a standard technique in microbiology for the disruption of living cells to release their contents. Indeed ultrasound has been studied as a possible method for water disinfection (36). Now evidence is emerging that controlling the power of sonication can lead to beneficial, non-lethal, effects on biological activity e.g. the promotion of bioreactor efficiency (37,38). In our experiments prolonged exposure of *P. putida* UWC1 to 40 kHz for more than 60 s efficiently killed bacterial cells (Figure 1B), which is to be expected as low frequency ultrasound has been used for cell lysis and disruption (39–41). Exposure times of less than 30 s did not significantly reduce cell viability (Figure 1B). Interestingly, exposure time of 10 s resulted in higher colony counts in viability assays, which may be due to the short ultrasound treatment separating and dispersing clumped cells (Figure 1B).

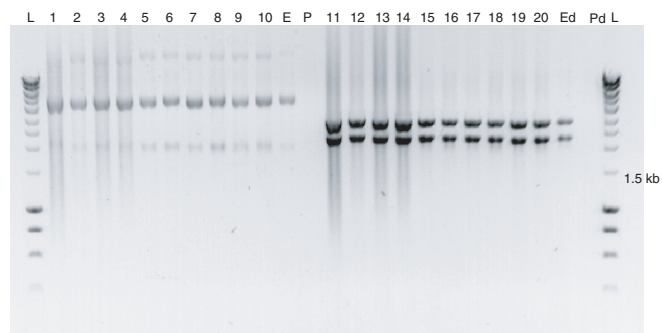
Exposure of plasmids to ultrasound for 30 or 60 s significantly damaged plasmid integrity and sheared plasmids into fragments (Figure 1C), which is in good agreement with observations carried out by Grokhovsky (42). However, plasmid exposure times less than 10 s did not affect plasmid integrity, as assessed by gel electrophoresis (Figure 1C).

Occasionally, plasmids with 30 and 60 s ultrasound exposures (Figure 1C, lanes 11 and 13) were not completely sheared; later examination indicated that in those cases the bottoms of vials and 375H were not parallel, and some the ultrasound energies might be reflected. Therefore, in order to efficiently transport ultrasound energy to the cell–plasmid mixtures, the flat bottom of vials should be parallel with the bottom of 375H ultrasonic cleaning bath.

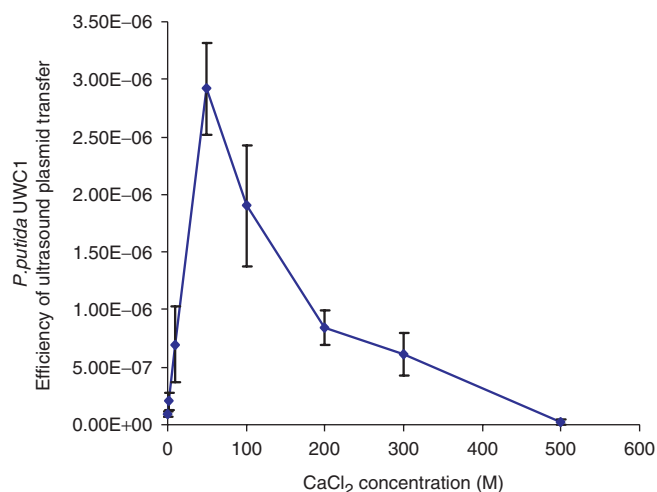
The results of cell viability and plasmid integrity assays are in good agreement with UDD result (Figure 1), which showed that plasmid transfer occurred at a high frequency following ultrasound treatments of 5–30 s. Since 30 s ultrasound exposure time damaged plasmid DNA (Figure 1C) and 5 s exposure time gave relatively variable results (Figure 1A), all subsequent experiments were performed with a 10 s ultrasound treatment.

#### Confirmation of plasmid delivery to *P. putida* UWC1, *E. coli* DH5 $\alpha$ and *P. fluorescens* SBW25

We randomly picked 10 ultrasound-transformants of each species: *P. putida* UWC1, *E. coli* DH5 $\alpha$  or *P. fluorescens* SBW25 to confirm that kanamycin resistance arose as a result of UDD. Ten transformants of each species were confirmed by 16S rDNA sequencing of each colony, which confirmed that all 10 transformants had identical 16S rDNA to *P. putida* UWC1, *E. coli* DH5 $\alpha$  or *P. fluorescens* SBW25. Plasmid DNA was extracted from each of the 10 transformants. The plasmids isolated from ultrasound transformants *P. putida* UWC1 (Figure 2), *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25 (data not shown) were digested with NotI and compared with pBBR1MCS2 and confirmed they had identical structures. Finally, the plasmids were partially sequenced and the results confirmed that they were pBBR1MCS2.



**Figure 2.** Confirmation of ultrasound-mediated delivery of pBBR1MCS2 into *P. putida* UWC1. L: ladder (BioLone, UK, HyperLadder I), E: pBBR1MCS2 extracted from *E. coli*, P: UWC1 control, Ed: pBBR1MCS2 digested with NotI, Pd: NotI digestion of plasmid extract from UWC1 control, 1–10: plasmid extracted from UWC1 transformants and 11–20: plasmids from UWC1 transformants digested with NotI.

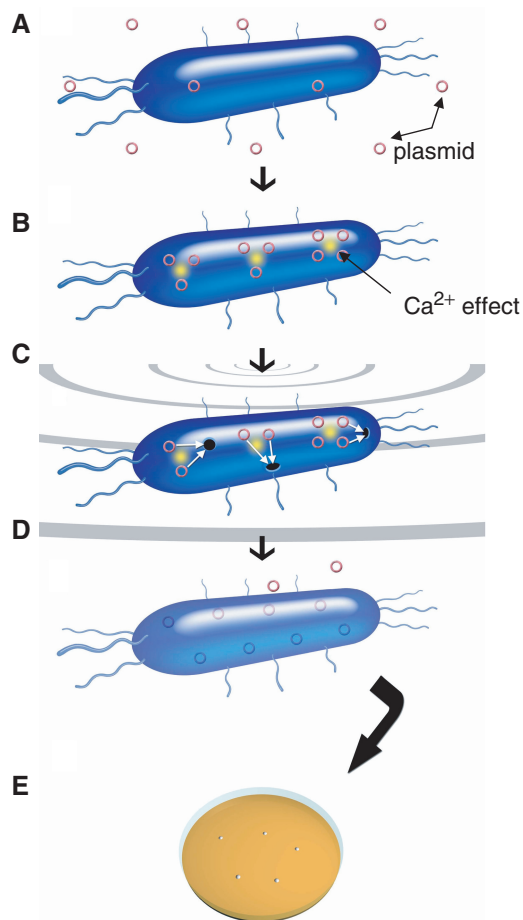


**Figure 3.** Calcium chloride promotes ultrasound-mediated transformation of *P. putida* UWC1. Addition of 50 mM CaCl<sub>2</sub> in transfer mixture gave the highest UDD efficiency ( $P < 0.05$ ).

#### Addition of calcium chloride to DNA transfer media significantly enhances UDD

We tested the effect of four different media on UDD efficiency, with *P. putida* UWC1 as the recipient strain: LB, phosphate buffered saline (PBS), MgSO<sub>4</sub> and CaCl<sub>2</sub>, respectively. Only CaCl<sub>2</sub> had a significant effect on UDD (data not shown). CaCl<sub>2</sub> concentrations lower than 10 mM or greater than 500 mM had no significant effect on UDD (Figure 3). The optimal CaCl<sub>2</sub> concentration for UDD was 50 mM CaCl<sub>2</sub> (Figure 3).

Langer and colleagues suggested that the mechanism of sonophoresis with low frequency ultrasound, such as 20 kHz, is primarily due to cavitation effects (43–45). We propose a mechanism of ultrasound-mediated plasmid transfer of bacteria in which plasmid transfer is enhanced in the presence of CaCl<sub>2</sub> (Figure 4): Plasmid DNA and bacteria are initially well-mixed in an aqueous solution (Figure 4A). The addition of CaCl<sub>2</sub> causes changes in the

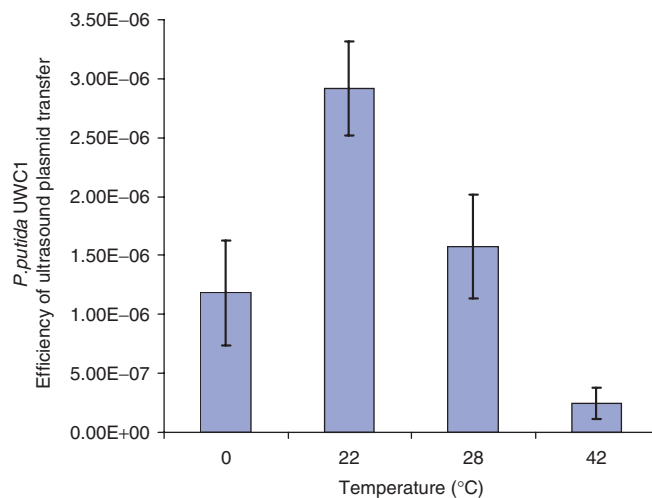


**Figure 4.** Illustration of proposed mechanism of ultrasound DNA transfer. (A) A bacterial cell and plasmid DNA are suspended in an aqueous solution. (B) 50 mM  $\text{CaCl}_2$  promotes transformation. (C) Ultrasound generates pores in cell membranes through which plasmids enter the cell. (D) Pores are closed and plasmids are retained in the cell. (E) Bacteria acquire new functions after taking up plasmid DNA.

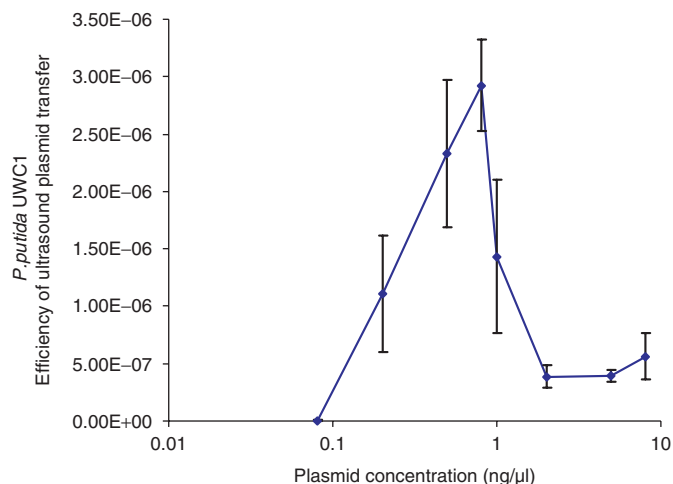
conformation of plasmid DNA or cellular membrane structures that promote transformation (Figure 4B). As low frequency 40 kHz ultrasound is applied to the solution the transmitted energy causes temporary porosity in the cell membrane, which enables the plasmids to enter through the pores (Figure 4C). When the ultrasound is switched off the cell membrane repairs itself and the transformed cell retains the plasmid DNA (Figure 4D). Bacteria acquire new functions, such as growing on a selective medium, after taking up plasmid DNA (Figure 4E).

#### Temperature affects UDD efficiency

The efficiency of UDD to *P. putida* UWC1 at 22°C was higher than UDD efficiency at 0, 28 or 42°C (Figure 5). However, the reason for this remains unclear. We monitored the temperature of DNA transfer media during 10 s ultrasound, and found that short-term ultrasound treatment did not increase the temperature of cell-plasmid reaction solutions (data not shown), which rules



**Figure 5.** Temperature effect on UDD efficiency of *P. putida* UWC1. UDD at 22°C gave the highest efficiency ( $P < 0.05$ ).



**Figure 6.** The effect of plasmid concentration on UDD of *P. putida* UWC1. High concentration plasmid reduced UDD efficiency ( $P < 0.05$ ).

out the possibility that thermal effects associated with ultrasound treatment affected UDD.

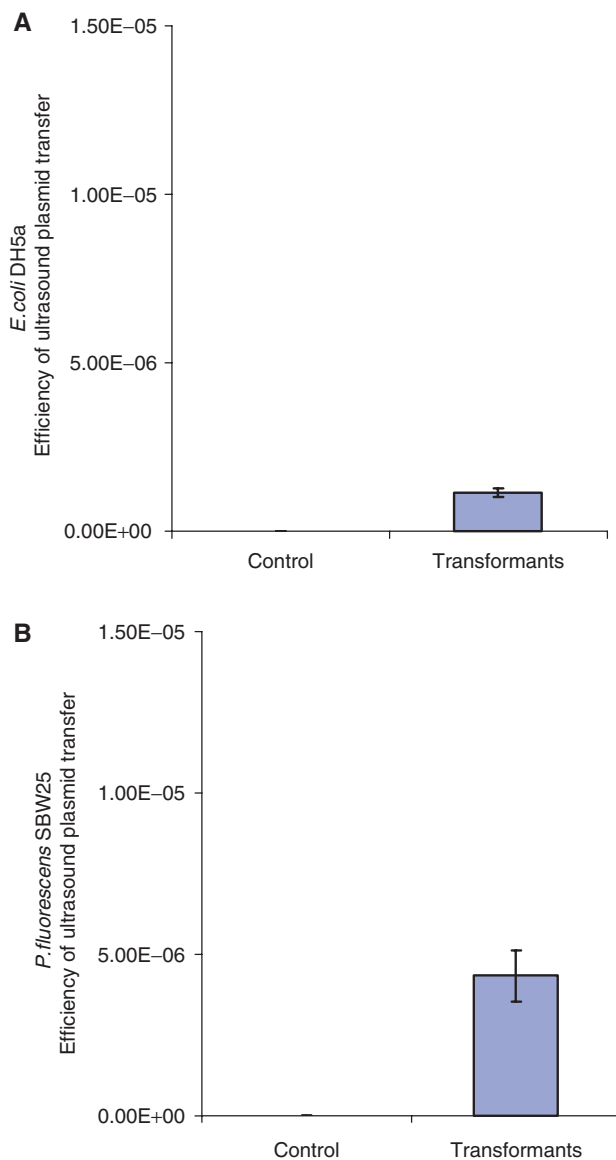
#### The effects of plasmid and cell concentration on UDD

UDD efficiency was highest at plasmid concentrations of 0.8 ng/μl with decreasing efficiency at lower and higher values (Figure 6). Cell concentrations of  $6.0 \pm 0.9 \times 10^7$  CFU/ml or less did not give a significant number of transformants at a plasmid concentration of 0.8 ng/μl (Table 1). However, increasing concentration of plasmids up to 8 ng/μl did generate a few transformants. Since UDD requires the interaction of cells and DNA, as cells concentration was low, the chance that plasmids meet cells significantly decreased. This may suggest that the number of collisions between cells and plasmids is important for UDD. Cell concentrations of  $5.3 \pm 1.3 \times 10^8$  and  $3.2 \pm 0.3 \times 10^9$  CFU/ml gave UDD efficiencies of  $1.1 \pm 0.4 \times 10^{-6}$  and  $4.2 \pm 2.4 \times 10^{-6}$  transformants per

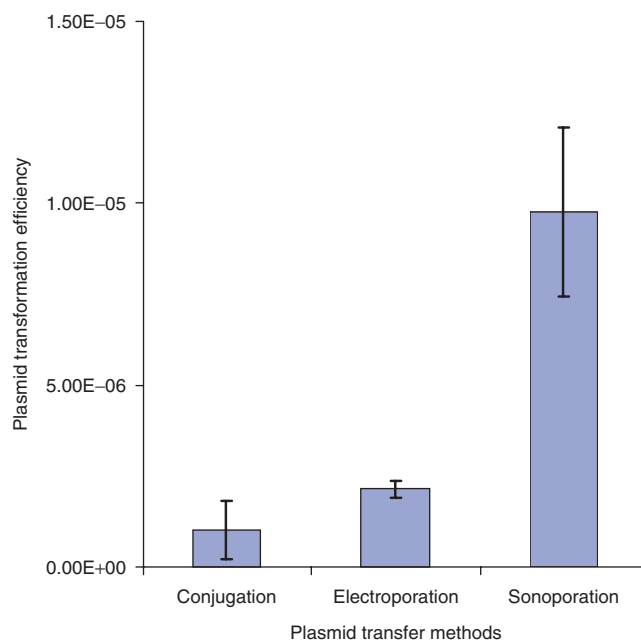
**Table 1.** The effect of *Pseudomonas putida* UWC1 cell concentration on ultrasound DNA delivery

Cell concentration (CFU/ml)	Transformation frequency (transformants per cell)
$6.04 \pm 0.92 \times 10^7$	0
$5.32 \pm 1.29 \times 10^8$	$1.14 \pm 0.44 \times 10^{-6}$
$3.22 \pm 0.34 \times 10^9$	$4.24 \pm 2.38 \times 10^{-6}$

pBBR1MCS2 final concentration was 0.8 ng/ $\mu$ l. Data shown is the average of 5 replicates  $\pm$  error.

**Figure 7.** Ultrasound DNA transfer can also be applied to *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25. (A) Ultrasound transferred pBBR1MCS2 to *E. coli* DH5 $\alpha$  ( $P < 0.05$ ) and (B) Ultrasound transferred pBBR1MCS2 to *P. fluorescens* SBW25 ( $P < 0.05$ ).

cell, respectively. These findings show that the efficiency of UDD in bacterial cells is affected by the cells population density and the concentration of plasmid DNA used during the sonoporation process.

**Figure 8.** Comparison of plasmid transfer methods. Ultrasound-mediated delivery can give a higher transformation efficiency than conjugation or electroporation ( $P < 0.05$ ).

#### UDD delivered plasmids to *E. coli* DH5 $\alpha$ or *P. fluorescens* SBW25

To ensure the 40 kHz ultrasonic cleaning bath can deliver DNA to *P. putida* UWC1 is not a single case, we have also attempted to transfer plasmids to *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25. Using the optimal conditions based on UDD experiments of *P. putida* UWC1, we have successfully delivered pBBR1MCS2 to *E. coli* and *P. fluorescens* SBW25 with efficiencies of  $1.16 \pm 0.13 \times 10^{-6}$  and  $4.33 \pm 0.78 \times 10^{-6}$  transformants per cell, respectively (Figure 7A and B). To improve UDD efficiency of *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25, more work in the future is needed, since the optimal conditions for *P. putida* UWC1 may not necessarily be the best for *E. coli* DH5 $\alpha$  and *P. fluorescens* SBW25E.

#### Comparison of conjugation, electroporation and UDD of *P. putida* UWC1

Under optimal conditions for *P. putida* UWC1: ultrasound exposure time of 10 s, 50 mM CaCl<sub>2</sub>, temperature of 22°C, plasmid concentration of 0.8 ng/ $\mu$ l, *P. putida* UWC1 cell concentration of  $2.5 \times 10^9$  CFU/ml and reaction volume of 500  $\mu$ l, the efficiency of UDD was  $9.8 \pm 2.3 \times 10^{-6}$  transformants per cell. UDD efficiency was compared with two traditional plasmid transfer methods: conjugation and electroporation, and was found to be nine times higher than conjugation and up to four times higher than electroporation (Figure 8).

The ultrasound apparatus used in this study was a standard ultrasound bath. Further investigation of the effects of ultrasound frequency, intensity, alignment and distribution on UDD may well lead to further

improvements in transformation efficiency, and allow this process to be scaled to an industrial level. Our results have shown that UDD is a viable technology for bacterial transformation, which is far less dependent on the ionic strength of the medium than electroporation, allowing it to be used with bacteria growing in complex environments and under more natural conditions. We believe UDD will open up numerous new opportunities for DNA and RNA delivery into a wide range of microorganisms.

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