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Frequency-dependent ultrasound-induced transformation in *E. coli*

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Abstract (140 words)

Ultrasound-enhanced gene transfer (UEGT) is continuing to gain interest across many disciplines; however, very few studies investigate UEGT efficiency across a range of frequencies. Using a variable frequency generator, UEGT was tested in *E. coli* at six ultrasonic frequencies. Results indicate frequency can significantly influence UEGT efficiency positively and negatively. A frequency of 61 kHz improved UEGT efficiency by ~70% higher, but 99 kHz impeded UEGT to an extent worse than no ultrasound exposure. The other four frequencies (26, 133, 174, and 190 kHz) enhanced transformation compared to no ultrasound, but efficiencies did not vary. The influence of frequency on UEGT efficiency was observed across a range of operating frequencies. It is plausible that frequency-dependent dynamics of mechanical and chemical energies released during cavitational-bubble collapse (CBC) are responsible for observed UEGT efficiencies.

Keywords: bacteria; plasmid; sonoporation; transformation; ultrasonic

Introduction

Bacterial genomes are fluid. The diversity and adaptability of microorganisms are due to their ability to acquire mobile genetic elements by horizontal (or lateral) gene transfer. This has been responsible for driving bacterial evolution, and it is currently exploited in biotechnology to manipulate metabolisms in microorganisms. Natural gene transfer between microorganisms is dependent on three mechanisms: transduction, transformation, and conjugation. The main thrust of this research was to determine whether horizontal gene transfer via transformation can be enhanced by use of ultrasonic energy.

It has been demonstrated that ultrasound exposure enhances the efficiency of gene transfer into bacterial cells in solution, and even induce transformation in non-competent cells (Song et al. 2007). Exposure to ultrasound temporarily, and reversibly, increases the permeability of cell membranes, allowing larger molecules such as plasmid DNA to pass through the cell membrane in a phenomenon known as "sonoporation". The increased permeability in cell membranes is largely the result of secondary forces generated by cavitation, the growth oscillation compression and explosion of micro-bubbles in solution (Newman and Bettinger 2007). These secondary forces generated by cavitation include extreme local

pressures and temperatures, which increase the membrane porosity, and micro-jets generated by bubble collapse can inject molecules (or plasmids) into the cell.

However, the mechanism by which ultrasound physiologically alters cells to enhance genetic transformation can also decrease cell viability. Ultrasound power intensity and period of exposure will influence the type and degree of bio-effects experienced by the cells. Therefore, with regard to genetic transformation, there exists a therapeutic ratio where enhancement of transformation efficiency is greatest and impact on cell viability minimal. Subsequently, researchers have attempted to optimize ultrasound operation by adjusting parameters such as acoustic power and duration of exposure (Song et al. 2007). Despite these efforts, the ultrasonic frequency, as a parameter of operation, has largely been overlooked. Previous studies have been limited by instrument availability, tending to operate at a single frequency and power (e.g., 20, 40 or 80 kHz).

It has not been determined whether the feasibility of plasmid incorporation is frequency dependent. Using a variable-frequency generator, we determined whether ultrasound-induced transformations are affected by frequency. The use of ultrasound to induce high rates of gene transfer has the advantages of not being media dependent and non-invasive. This opens technological applications requiring a transformation strategy not bound by electro-chemical requirements.

Materials and Methods

Ultrasound (US)-system setup consisted of a custom-built 2.54 cm diameter Tonpilz probe-type transducer connected to a function/arbitrary waveform generator (Agilent, model 33220A, 20 MHz capacity), an amplifier (Kalmus, model 155LCR), and a custom-built impedance-matching circuit, between the amplifier and transducer, to increase power output. Acoustic power was determined by radiation force balance (Rooney 1973), and adjusted to achieve an intensity of 0.240 W cm^{-2} at all operating frequencies.

Samples consisting of 5.0 x 10^{10} CFU ml^{-1} *E. coli* MC1000 cells and 0.1 ng μl^{-1} plasmid DNA [(pGFPuv (Clontech Laboratories, Mountain View, CA; GenBank Accession #U62636), 3.3 kbp: pUC, *lacZ*-GFPuv, amp^r) in TE buffer (pH 8.0)] re-suspended in phosphate buffered saline (PBS) buffer [all PBS buffer was pH 7.0 and room-temperature (22 °C)] to a total volume 10 ml, were poured into a sonication-container —a

closed bottom, clear plastic Perspex tube (5 cm inner-diameter and 1 mm thick walls)—for US exposure (sonication). The transducer tip was submerged into a sample-suspension (US medium), directing the beam downward into the sample, which was sonicated for 10 s (single-pulse) at room temperature (22 °C) with an operating frequency of either 26 kHz, 61 kHz, 99 kHz, 133 kHz, 174 kHz, or 190 kHz. Five replicate experiments (samples) were conducted at each frequency and for the control group (no US exposure), and means ±SE (standard errors) were reported.

Before exposure, cells grown overnight (17 hrs in 100 ml LB broth, 37 °C with 150 rpm shaking) were harvested by centrifugation (7000 rpm at 4 °C for 5 min), washed twice with 30 ml PBS buffer, re-suspended in 10 ml PBS buffer in 50-ml polypropylene conical-bottom centrifuge tubes, and left for (1.5 h) to reach room temperature (22 °C). Plasmid DNA was extracted and purified from overnight cultures of transformed *E. coli* MC1000, a few days prior to UEGT experiments, using QIAGEN Plasmid Maxi Kit. Cell and plasmid concentrations were measured using a BioTek Epoch Micro-Volume Spectrophotometer (OD600 nm and OD260 nm, respectively).

Plasmid DNA were added to each sample 60s prior to exposure. Samples were mixed in the 50 ml centrifuge tubes by inverting 6 times, and then poured into the SC. After US exposure (sonication), samples were returned to 50 ml centrifuge tubes, incubated 1h at room temperature (22 °C), after which serial dilutions were prepared (in PBS) and plated onto selective LB agar (100 μg ml^{-1} ampicillin) to screen for transformants. SC and transducer tip were cleaned between samples with 70% ethanol, rinsed twice with dH_2O, once with PBS, and dried. After 24 h incubation (37°C), CFU's were counted, GFP (green fluorescent protein) gene expression in select colonies was confirmed via fluorescent microscopy (Olympus BX60).

Results and Discussion

E. coli cells were exposed to six ultrasound frequencies (26 kHz, 61 kHz, 99 kHz, 133 kHz, 174 kHz and 191 kHz; all at 0.240 W cm^{-2}), generated from a variable frequency generator, and pGFP plasmids (pUC, *lacZ*-GFPuv, amp^r). The control group (sans sonication) did produce some transformants, indicating a degree of pre-existing competency and providing a baseline for UEGT efficiency comparison. Differences in transformation rates were noted once cells were exposed to plasmids in presence of US. Comparison of

means ± SE from each frequency and the control group are presented in Table 1. One-way *ANOVA* test revealed frequency significantly influenced UEGT efficiencies ($F_{6, 28} = 91.3$, $P < 0.001$). Post-hoc analyses via Tukey's HSD confirmed CFU-means from all frequencies varied significantly from the control group ($0.000 < P < 0.003$). However, of the six frequencies tested (Figure 1), only two, 61 kHz and 99 kHz, produced significantly different results from the other four, 26 kHz, 133 kHz, 174 kHz, and 190 kHz ($0.000 < P < 0.001$)—among which there were no significant differences ($0.469 < P < 1.000$).

TABLE 1 … FIGURE 1

Absence of growth on selective media (100 μg ml^{-1} ampicillin) from MC1000 cells, at the same concentration but unexposed to plasmid DNA, ruled out natural antibiotic resistance as a factor in microorganisms' growth in control treatment, and confirmed pre-existing competency in the control group. The observation that five of the six frequencies investigated produced more CFU than the control group supports previous assertions (Song et al. 2007) that UEGT can improve bacterial transformation efficiencies. Maximizing UEGT efficiency, however, was not the focus of this study, but rather to investigate the influence of frequency as a single-parameter on UEGT efficiency in bacteria. For the first time, evidence presented herein indicates significant influence of operating frequency in UEGT efficiency.

At lower frequencies, individual cavitation-bubble collapse (CBC) events release more energy and occur less frequently due to larger bubble growth, resulting in greater spatial and temporal concentration of mechanical effects from each CBC than at higher frequencies (Mason et al. 2011). Implications for physical modification of surface materials are described by Mason et al. (2011) who observed, via Scanning Electron Microscopy (500x), more distinct, though spatially less uniform, physical surface-modification—visually recognizable at the 1-μm level—of plastic wafers at 20 kHz and considerably less obvious, though more uniformly distributed, physical modifications at 40 kHz despite similar sums of net mechanical impact. Likewise, Tezel et al. (2001) found exposing skin to 20 kHz generated macroscopically larger, more spatially concentrated pores than smaller, more spatially disperse pores produced at 58.9 kHz despite equal increases in net permeability (electrical conductivity) from both. Furthermore, during cellular sonoporation each incidence of cell membrane-perforation (pore formation) is largely attributable to the jetting force (micro-jets) from an individual CBC, as revealed by high-speed photography (Kudo et al.

2009, Ohl et al. 2006), and it has been demonstrated pore size can vary considerably, e.g. 20–1000 nm, by changing operating parameters other than frequency (Newman and Bettinger, 2007, Schlicher et al., 2006).

Collectively, this evidence (e.g., Mason et al. 2011, Newman and Bettinger 2007, Ohl et al. 2006, Schlicher et al. 2006) suggests frequency could similarly influence properties of pore-morphology during sonoporation, e.g. pore-size, shape, quantity, location and/or density on cell membranes, even if net-changes to membrane permeability are frequency-independent. Since CBC jet-force magnitude is inversely related to frequency (Mason et al. 2011), we can logically assume characteristics of subsequent pore morphology, e.g. size and expanse, are too. Therefore, it is plausible membrane-disruptions (pores) were more severe at 26 kHz compared to more frequent, though less severe disruptions, at 61 kHz, which could affect UEGT efficiency.

Sufficient membrane-disruption(s) is essential for UEGT, but cell death ensues if too severe (Joyce et al. 2011). If characteristics of membrane disruption(s) are frequency-dependent, an optimum frequency likely exists for maximizing UEGT efficiency. More severe disruptions at 26 kHz than 61 kHz could have disfavored cell repair and viability equating to lower efficiency at 26 kHz than 61 kHz (Figure 1). At higher frequencies, spatial and temporal de-concentration of mechanical-effects from individual CBC-events (Mason et al. 2011, Tezel et al. 2001) could have induced sub-optimal sonoporation qualities, i.e. insufficient permeabilization, for plasmid uptake or UEGT, equating to higher efficiency at 61 kHz than at 133 kHz, 174 kHz, and 190 kHz.

The extremely low efficiency at 99 kHz does not seem to fit this proposed mechanistic-influence of frequency. Significant differences in the acoustic power absorbed by the medium in the sonication chamber at various frequencies investigated were ruled out by calorimetry (Margulis and Margulis 2003), and although free radical oxidation can be a major cause of supercoiled plasmid DNA degradation in pharmaceutical formulations (Evans et al. 2000), exposing plasmid DNA (suspended in TE buffer pH 8.0) to all frequencies used produced no noticeable changes in plasmid-integrity, as revealed by gel electrophoresis (data not shown). Collectively, this suggests some additional factor(s) can strongly influence UEGT efficiencies, if only at discrete frequencies.

The influence of specific ultrasonic frequencies on sonolysis of water into H^+ and oxidative OH^- radicals, affects species compositions and secondary product formation, e.g. H_2O_2 (Hua and Thompson 2000). Consequently, an optimum frequency exists at which sonochemical reactions with OH^- are maximized, determined by their physical and chemical properties (e.g., Jiang et al. 2006), and specific US frequencies can influence biomolecule ionization(s) (Wu et al. 2010). Additionally, it's been demonstrated extracellular Ca^{2+} is a dominant factor in membrane-recovery after sonoporation (Kudo et al., 2009, Kumon et al. 2009), implying potential sonochemical-modulation of membrane-repair. Subsequently, it is plausible certain frequencies might uniquely affect enzymatic, biomolecular, and cellular activities and functions, e.g. transcription and membrane-repair, through sonochemical modification(s) and/or biochemical cascades associated with UEGT.

The potential for free radical formation to impede cellular function and/or UEGT at specific frequencies could explain why 99 kHz was the only frequency producing less CFU than the control group and why efficiency at 99 kHz was so low (Figure 1). If sonochemistry strongly influenced efficiency at 99 kHz, then we cannot rule out the role of free radicals at other frequencies. However, since free radical production is favored at higher versus lower frequencies and concentration in the medium correlates positively to frequency (Mason et al. 2011), it is reasonable to suspect that, if free radicals were the dominant efficiency at most frequencies, increasing free radical concentrations via higher frequencies, i.e. from 133 kHz to 174 kHz to 190 kHz, should correlate to greater influence, i.e. further loss of efficiency. As this was not the case (Figure 1), it is unlikely sonochemistry was the dominant aspect in most frequencies.

In addition, similarity of efficiency ($P \geq 0.999$) at these three frequencies (133 kHz, 174 kHz, and 190 kHz) indicates some stabilization of UEGT efficiency (Figure 1), suggesting the impact of frequency may be less substantial at higher versus lower frequencies. This supports the assertion the influence of frequency in UEGT is more likely attributable to changes in mechanical energies rather than greater production of free radicals, which occurs higher vs. low frequencies (Mason et al. 2011). However, since free radicals were not explored in our study, no logical conclusions can be drawn concerning their role(s) in observed trends. Also, as Newman and Bettinger (2007) point out, the role of free radicals in UEGT remains controversial.

Ultimately, our data seemingly suggest that the influence of frequency on UEGT is largely mediated by the frequency-dependent nature of mechanical effects of CBC, though at discrete frequencies sonochemical aspects might dominate UEGT efficiency instead. It is possible the influence of frequency might be less substantial across a higher versus lower frequency-range, but further investigation is needed to draw any logical conclusion(s) concerning dynamics between frequency-dependent chemical and mechanical effects of CBC, sonoporation qualities, and bio-effects posited herein. However, as this is the first time frequency has been observed to influence UEGT-efficiency across a range of operating-frequencies, this work should be considerably valuable to the rapidly expanding use of ultrasound in microbiological applications of various disciplines and to understanding bio-cellular response to US exposure. Future studies could also benefit by analyzing frequencies' effects, e.g. ionization dynamics, on media and buffers - particularly those containing salts and metallic elements, e.g. PBS and their constituents, used in UEGT which could affect plasmid integrity (Wu et al. 2010, Evans et al. 2000).

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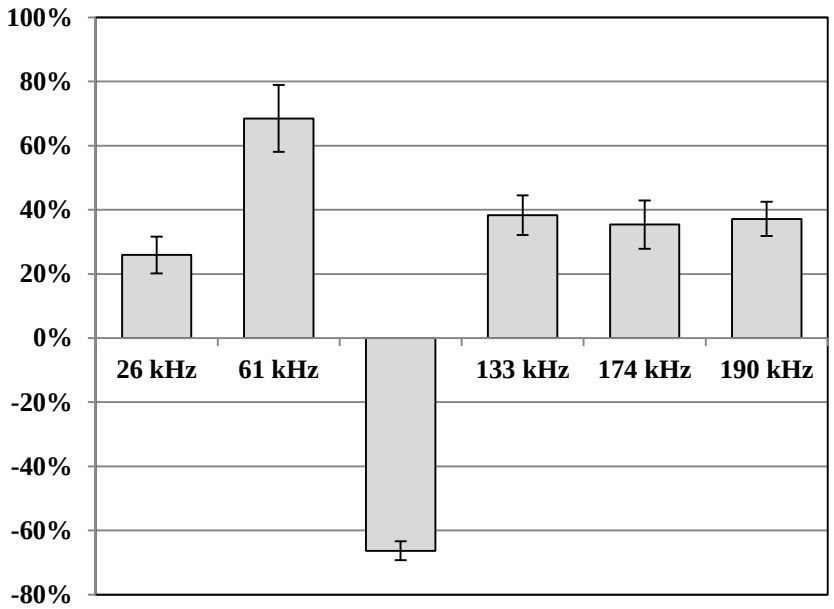
Table 1. Number of transformants recovered following ultrasonic treatments at different frequencies.

Treatment	Mean ($\times 10^7$ CFU)	(Standard Error)
26 kHz	4.3	(0.2)
61 kHz	5.7	(0.6)
99 kHz	1.1	(0.2)
133 kHz	4.7	(0.3)
174 kHz	4.6	(0.4)
190 kHz	4.6	(0.2)
Controls (no US)	3.4	(0.3)

Figure Caption:

Fig 1. Percent increase of transformation rate, as compared to no-ultrasound controls. Vertical bars represent ± standard error.



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