Ultrasound-mediated Gene Transfer to Enhance Bioremediation of Contaminated Water

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Abstract

A novel technique for *in situ* bioremediation is vital to enable the world to meet the need to treat contaminated land; ultrasound gene transfer has that potential.

Ultrasound gene transfer has been shown to be a non-invasive, low impact and practical engineering method to deliver plasmid DNA and macro-molecules into bacteria. For the first time delivery of a salicylate hydroxylase gene into *P. putida* UWC1 has been demonstrated, enabling the complete degradation of the salicylate contaminant, which the wild type was unable to degrade. Furthermore not only DNA but also macro-molecules (e.g. fluorescent tagged large dextran molecules, up to 2,000,000 MW) have been delivered into *P. putida* UWC1 using UGT. This can potentially enable delivery of bioparts and nanomaterials for synthetic biology to targeted locations in an organism.

To achieve this, a novel variable frequency ultrasonic generator has been developed to deliver focussed ultrasound through the sonotrode directly into an aqueous bacterial sample. This sonotrode was designed to operate at the optimum frequency for UGT of 27.5 kHz determined using the preliminary apparatus and has enabled the application of UGT to ~50 ml samples, demonstrating scalability to industrial application (i.e. using an array of sonotrodes to treat litres of environmental sample for re-introduction). The optimum frequency enables a satisfactory rate of transfer (10^{-7} efficiency) whilst minimising cell lysis (<90% cell survival) making it ideal for environmental application as it will minimise unnecessary disruption to the ecosystem.

The mechanism behind UGT has been determined as transfer peaks at the resonant frequencies where cavitation microbubbles are produced. It is the collapse of these microbubbles that generates microjets of extremely high pressure that affect the cell walls of the bacteria enabling uptake of the DNA or macro-molecules.

Thus it is shown that the emerging technology of ultrasound gene transfer can deliver novel genes directly into bacteria with minimal preparation and minimal impact to the cells.

Acknowledgements

After eight years, with much fun and adventure, countless friends, colleagues and acquaintances, my time in the Steel City is coming to an end. It is a sad time but also a new beginning and a chance to reflect on all who I have met and who have helped shaped me throughout my time here, from academic course mates and tutors, sporting team mates and organisers and the varied housemates that have added such colour to my life in this wonderful place. Thank you.

Many thanks to Wei, for your endless enthusiasm and countless ideas, I'm sure you and your students will continue to achieve great things. Emma, Paul, Harry, Tom, Michael and the all others in the KRI your support has been invaluable whether technical advice or just a friendly face when needed, your company over these 4 years has been a pleasure and I look forward to sharing a beer with you all for years to come.

To my friends and family that have supported me over the last few weeks, thank you for the encouragement and the comfort food that has kept me going to the end, I looking forward to returning the favours in the future.

Now I must step out into the world of industry but will never forget these wonderful years at the University of Sheffield and all of you who have made it special.

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Section 1: Introduction and Scope of Research

1.1: Background and Premise for this Research

…to develop a non-invasive low impact solution

to introduce pollutant degrading genes

in situ *into indigenous bacterial communities…*

With millions of polluted sites across the globe solutions are needed to decontaminate them, current remediation techniques whether physical, chemical or biological have proven to be limited by: impractically for *in situ* use, expense (especially *ex situ* treatments), lengthy timescales (natural attenuation) and in some cases actually further damage the ecosystem. Existing biological treatments can also fail to adapt to the environmental conditions or can introduce alien species so the ability to use the indigenous species is a key driver in development.

There are many current techniques for bacterial gene transfer however they are all laboratory based and restricted to individual cells or microlitre volumes of carefully prepared single culturable species. Moreover these carefully manipulated cells can rarely survive when applied to environmental conditions, especially polluted sites. Therefore the capability to introduce genes to degrade these pollutants *in situ* into indigenous communities cannot be undervalued.

It is hypothesised that this research can demonstrate that **ultrasonic gene transfer** can provide a novel solution with the potential for *in situ* enhanced natural bioremediation without introducing foreign species or harmful chemicals. The main aim will be to focus on the microbiological techniques and application for bioremediation and the interaction between these to create a powerful application to meet these needs. Concurrently the work will also assess the scope for application of ultrasonic gene transfer to a plethora of industrial applications from creating microbial chemical factories to improving microbial fuel cells and beyond to ensure the potential of this embryonic technique can be realised.

1.1.1: Contaminated Environments; Bioremediation, Bioaugmentation and Biostimulation

Worldwide pollution is a major concern, both decontaminating existing sites and treating waste to prevent new pollution events. In recent years there has been large investment into improving remediation and minimising environmental damage however despite this there is increasing demand for stricter controls on industry requiring better treatment of effluents and increased urgency to clean previous spills.

These pollutants come from a wide range of sources, both diffuse and point sources, and are made up of a variety of compounds, from hydrocarbons and solvents to heavy metals and pesticides, as a result the contaminant treatment is a difficult and often expensive process, with current estimates being in excess of £11.5 billion in the UK (CIRIA, 2011) and $1.7 trillion in the USA (Van Dillewijn et al., 2009).

Focussing on the UK, there is estimated to be over 300,000 hectares of contaminated land and a corresponding amount of ground water contamination across the country. This had led to the Environment Agency classifying over 7% of all current groundwater abstraction for drinking water as unusable (EA, 2006) and despite the rainfall across much of the UK some areas now have less available water per person than found in Syria (Bourn, 2005); this shortage has led to regular water restrictions over summer periods, especially in the south and east of England. Increased demand for brownfield land in previously industrial city centres means decontamination of this ex-industrial land is becoming more pressing. Here it can be clearly seen the wide range of potential demand for an *in situ* cheap and simple bioremediation technique to flourish.

Currently to treat these contaminated areas there are three main types of remediation: chemical, physical and biological. Chemical treatments involve dosing the ground with compounds to: reduce the toxicity by surface complexation causing ionic or phase changes, immobilise the contaminants by sorption into a substrate or improve microbial degradation (e.g. electron acceptors, oxidising agents and nutrients). Chemical treatments can be expensive and only last a short period of time on sites where continuous contamination occurs as they can be quickly used up requiring repeat doses. Furthermore there is also the risk of further contaminating the ecosystem by overdosing with chemicals and creating more damage either through

poisoning or eutrophication. Physical treatments include soil washing, aeration and sparging; these are very labour intensive and can be disruptive to the surroundings especially if soil is removed for *ex situ* treatment. Finally current biological techniques include the use of plants (phytoremediation), fungi (mycoremediation) and bacterial/microbial treatments using bioaugmentation and/or biostimulation to accelerated contaminant degradation or to improve immobilisation of the pollutants by sorption into the added organisms; these have the scope to be most effective for the lowest impact and cost. Many biological treatments are, however, still in their infancy: there are some pollutants they are currently unable to degrade or immobilise and unknown impacts of introducing foreign species to an ecosystem.

Looking closer at bioremediation: it can be performed *in situ* in isolated areas as heavy machinery is generally not required, it also provides a long term treatment solution as the plants, fungi or bacteria used will remain in place and continue to break down further pollutants. The wide range and adaptability of organisms means that natural pathways occur for a large range of organic pollutants which can be degraded into harmless by-products or even used as energy sources by the degrader or other species and they can also immobilise heavy metals, retaining these in their cells or root systems.

Bioremediation has two main methods: bioaugmentation and biostimulation. Bioaugmentation is the addition of exogenous species to the polluted ecosystem to improve remediation. The introduction of non-indigenous species in bioaugmentation is also imperfect as the species introduced can either fail to adapt to the environment and perish or they become dominant and overwhelm the existing species, ultimately causing more long term damage if an invasive species is introduced to the ecosystem. Biostimulation is the modification of the existing environment to enhance the degradation of existing species (i.e. providing nutrients to boost growth or catalysts for degradation); these however generally involve chemical or physical treatments, which have the disadvantages shown above.

It can be seen that almost all treatments have the potential to harm the existing ecosystems, by introducing alien species or chemicals, negating any benefit gained from removing the pollutants; therefore when designing treatment projects any

additions should be minimised and their impact on the complex and interlinked ecosystems carefully analysed.

To avoid this UGT aims to provide: an *in situ* technique to enhance the degradation capabilities of indigenous species by only introducing specific degrading genes, introducing no alien species and adding no harmful chemicals solely improving the existing the existing organisms with plasmid DNA.

1.1.2: Unculturable Micro-organisms and the Requirement for a Culture Independent Gene Transfer Method

A major impediment to environmental microbiology is that, as it is widely recognised, over 99% of all micro-organisms cannot be cultured under laboratory conditions (Riesenfeld et al., 2004); this is due to specific nutrient and growth requirements that have not been identified or symbiotic relationships with other species that cannot be replicated in a laboratory. However, it is increasingly evident that as yet uncultured bacteria play key roles in biodegradation and bioremediation of environmental pollutants (Huang et al., 2009, Chen et al., 2010, Wang et al., 2012).This inability to culture species prevents the identification of these key degraders and hence these species and their genes cannot be targeted and harnessed. So to maximise the identification of potential degraders and the degrading genes culture independent techniques are required. These techniques include carbon-13 or other isotopic labelling of contaminants to identify, mark and trace the species being used to degrade them (Radajewski et al., 2000). Stable Isotope Probing (SIP) uses heavy isotopes to label a compound which is then used by a species, this is then incorporated into the DNA of cells that are able to utilise this compound. These can then be separated by ultracentrifugation and the DNA sequenced to isolate the different genes. Once isolated these can be replicated and inserted via a vector into other species to confer degradation ability to them, however current transfer techniques have many limitations (see Section 1.1.3), notable most are of either single cellular transfer or require culturing, where UGT is a large scale culture independent technique. Stable isotope probing and rapid gene sequencing techniques are quickly developing and the costs are dropping significantly, from the multi-billion dollar cost of the first human genome down to under $1000 (Begley, 2012) and individual gene synthesis has dropped over ten-fold since the mid ‘90s (Carlson, 2011), this has meant that culture independent techniques are becoming increasingly

accessible and media based culturing will soon become outdated for except for use in basic tasks.

Despite these advances a method for genetic manipulation of these unculturable micro-organisms has not been developed, and specifically not one for *in situ* usage. UGT has the potential to be this method: UGT does not rely on laboratory culture and as a mechanical transfer technique could, with the right ultrasound parameters, be applied to any bacterial species *in situ*, included unculturable strains, without the need for prior manipulation. Combined with cheap gene synthesis where degrading genes can be sourced, primers generated and inserted into plasmids for rapid multiplication, UGT can provide a cheap, simple and effective breakthrough technology in environmental bioremediation.

1.1.3: Current Gene Transfer Methods

The transfer of genes between bacteria is already widespread, occurring both in the environment as well as via mature laboratory techniques. However natural transfer is a random process and often has restricted donors and recipients. Laboratory techniques required specific environmental and chemical conditions, manipulation and are performed on small scales.

The natural inter-species transfer of DNA occurs regularly through a series of mechanisms, collectively known as Horizontal Gene Transfer (HGT). These include conjugation, transformation and transduction using plasmids and integrons. This allows for accelerated evolution and adaptation of species to their environments and environmental changes faster than the rate of change found in hereditary evolution (Johnsborg et al., 2007). It has been suggested that "the genomes of all bacteria can be considered as a single global gene pool into which most, if not all, bacteria can dip for genes necessary for survival" (Chen et al., 2005), this has been confirmed by Nakamura *et al.* (Nakamura et al., 2004) who have shown that 14% of open reading frames in the 116 prokaryotic genomes analysed had been recently horizontally transferred. The most commonly transferred genes included cell surface structure, biosynthesis, degradation, pathogenicity and antibiotic resistance genes (Nakamura et al., 2004); this explains how bacteria adapt so quickly to changing conditions and 'superbugs' rapidly develop resistance to antibiotics. The most common transfer methods are outlined here:

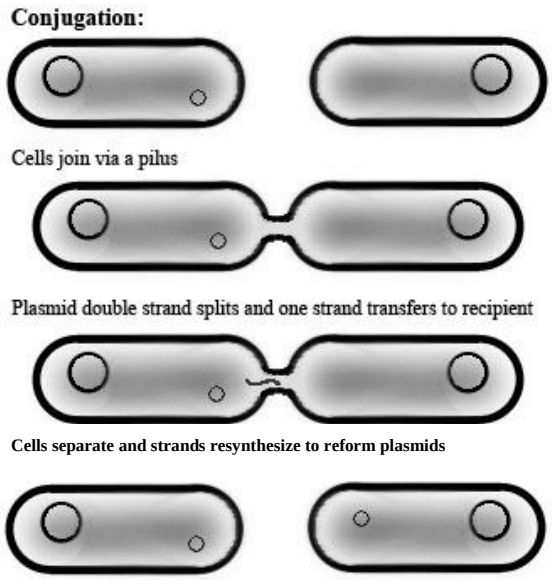

Figure 1: Conjugation of Bacteria

Conjugation is a plasmid coded mechanism involves cell to cell contact to transfer copies of the plasmid to a recipient cell (see Figure 1) and in some cases genetic elements that are un-transferable are also mobilised. First the cells link via a pilus, one strand of the donor plasmid transfers to the recipient whilst being replicated in the donor cell, this is then replicated in the recipient so both cells contain a complete plasmid, following synthesis the cells separate (Madigan M T, 2010, Davison, 1999, Chen and Dubnau, 2004, Chen et al., 2005, Lorenz and Wackernagel, 1994).

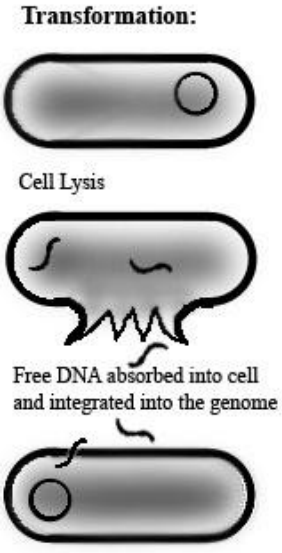

Figure 2: Natural Transformation of Bacteria

Transformation is the incorporation of free DNA into a recipient cell (Madigan M T, 2010, Davison, 1999, Chen and Dubnau, 2004, Chen et al., 2005, Lorenz and Wackernagel, 1994). When a cell is lysed the contained DNA is released and due to its fragile nature it breaks into small sections (approx. 10 kb). These sections can then be absorbed into the recipient cell, however not all cells can accept DNA in this manner, those which are able, are known as competent cells as they bind the free DNA onto the cell wall and either both strands are taken up or one strand is degraded by nuclease and the other taken up. The genetic information is then integrated into the genome by homologous recombination (see Figure 2).

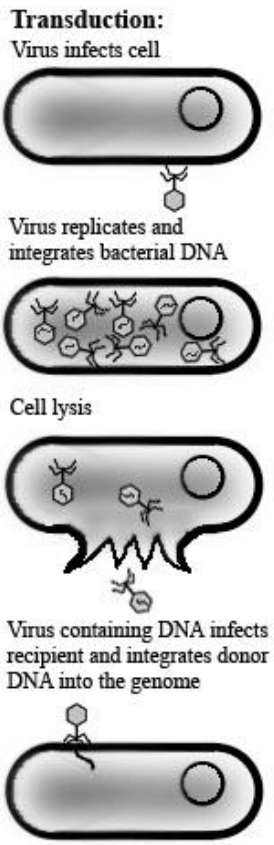

Figure 3: DNA Transduction Mediated by Viruses

Transduction occurs when a viral phage packages part of the host DNA into the virus genome (see Figure 3), when this then infects another cell the bacterial DNA is introduced and inserted into the genome by homologous recombination (Madigan M T, 2010, Davison, 1999, Chen and Dubnau, 2004, Chen et al., 2005, Lorenz and

Wackernagel, 1994). This is generally restricted to a narrow host range occurring between closely related species.

Whilst these natural techniques occur widely in the environment reproducing them to transfer specific genes under laboratory or industrial scales is more complicated. The majority of techniques revolve around transformation; however there are methods that use the mechanisms found in conjugation and transduction to mediate transfer.

Laboratory techniques offer a range of alternative ways to introduce DNA into bacteria, unfortunately current laboratory techniques have critical constraints that prevent them from being scaled to industrial or being applied *in situ*. The review below shows the most common laboratory techniques and their associated constraints:

Electroporation is one of the most commonly used techniques to introduce genetic information into bacterial cells. An external electrical field is applied to the cells suspended in a very high purity non-ionic solution. This creates an electro-magnetic field in the suspension which increases cell membrane permeability allowing large molecules or DNA to enter the cell. This is followed by a recovery phase in a high nutrient solution. The electrical field must be carefully controlled, too high and irreversible poration will occur lysing the cells but too low and the pores created will be insufficient, this however varies between species (Fiedler and Wirth, 1988, Yoshida and Sato, 2009); also if the solution contains too many ions the electrical field can arc between the electrodes killing the cells.

Due to the necessity for such specific conditions this procedure is limited to laboratory scale (µl) and medical work for small batch transfers. The efficiency and reliability of transfer is high so for small scale this is a very useful tool.

Recently attempts have been made to apply the theory to large scale experiments with some success. Artificial lightning (Ceremonie et al., 2006, Kotnik, 2013) has been applied to the surface of a soil sample and two strains have been successfully transformed in the laboratory and field scale work is on-going.

Heat-shock transfer uses the reaction of heat shock proteins in the cell wall to rapid changes in temperature to create pores in the cell wall. The cell suspension is cooled, rapidly heated (approx. 40°C), then re-cooled on ice (optimum temperatures do vary between species). The rapid temperature increase causes pores in the cell wall to opening allowing the genetic information in, and return to the cold closes these again, however as with electroporation these changes can irreparably damage the cell structure (Van Die et al., 1983, Yoshida and Sato, 2009).

Whilst this procedure does not rely on any specific chemical conditions in the solution, the ability to economically and practically heat and cool large volumes rapidly renders this method unsuitable for large scale use.

Biolistics and **needle injection** both insert genetic information directly into the cells on an individual basis resulting in very high efficiency and precision but this is a very laborious and time consuming operation. Biolistics use particles covered in the DNA and fires them into the cells and needle injection relies on insertion directly into the cell and the associated difficulty of accurate work on such a microscopic scale without permanently damaging the cells, thus it is predominantly used in larger mammalian cells (Yoshida and Sato, 2009).

High frequency ultrasound in mammalian cells is a relatively new technology but has gained popularity as it is simple to use and utilises currently available medical equipment. Transfer has been demonstrated in a range of cell types (Karshafian and Burns, 2009, Mehier-Humbert and Guy, 2005, Newman and Bettinger, 2007, Marmottant and Hilgenfeldt, 2003, Liu et al., 1998, Wyber et al., 1997, Save et al., 1994, Save et al., 1997, Kim et al., 1996, Deng et al., 2004, Bao et al., 1997, Miller et al., 1999, Zhou et al., 2009) with most work using Chinese Hamster Ovary (CHO) cells, however whilst laboratory work has been promising, *in vivo* efficiency is low (Kim et al., 1996).

This method uses the ultrasonic waves to create pores in the cell walls allowing the genetic information through, and whilst no specific chemical conditions are required the high nutrient and concentrations in laboratory testing have a markedly higher efficiency. However the more robust cell walls found in bacteria mean high frequency ultrasound has been markedly less effective.

Laser induced transfer has also been trialled for medical applications (Terakawa et al., 2004) with some success however it is still under development but looks to be an improvement on the biolistics by enabling less manipulation and larger sample numbers per transfer.

The calcium effect is used in many of the existing transfer techniques to improve efficiency of transfer. It has been found that concentrations of $CaCl_2$ of 50-100 mM have increased the rate of transformation of bacteria (Mandel and Higa, 1970, Yoshida and Sato, 2009). This effect occurs as the $CaCl_2$ aids the binding of free plasmids to the surface of the bacteria by de-polarising the cell wall prior to application of the transfer mechanism.

Table 1: Advantages and Disadvantages of Current Gene Transfer Methods (see section 1.1.3 for discussion and references)

Method	Efficiency	Volume	Cell Damage	Chemical Conditions	Temperature Restrictions	Sample Preparation	Wide Range of Recipient Species	Targeted Species/cells	*In Situ* application	Reference
Natural Transformation	Low	None	None	None	None	None	All	Random/ uncontrolled	Yes	(Chen et al., 2005, Nakamura et al., 2004)
Heat Shock	Medium	~50µl	Low	Medium	Specific heating and cooling	Competent cells and precise conditions	Competent cells	Single species	Laboratory only	(Van Die et al., 1983, Yoshida and Sato, 2009)
Electroporation	High	~50µl	High	Salt free	None	Pre-washing, salt free	Limited	Single species	Laboratory only	(Fiedler and Wirth, 1988, Yoshida and Sato, 2009)
Biolistics/ Needle Injection	High	Individual Cells	High	Low	None	Precise manipulation	Eukaryotes (and large cells)	Single cell	Laboratory only	(Yoshida and Sato, 2009)
Laser-induced	Medium	Micro-scale	Medium	Medium	Some heating	Targeting and manipulation	Eukaryotes	Single cell/tissue	Laboratory only	(Terakawa et al., 2004)
High Frequency Ultrasound (medical)	Low	None	Medium	Low	None	Minimal	Eukaryotes	Single species	Laboratory only	(Bao et al., 1997, Kim et al., 1996, Newman and Bettinger, 2007, Wyber et al., 1997)
Chemical (e.g. $CaCl_2$ Mediated)	Low	None	Low	Low ($CaCl_2$)	None	Minimal	Many	Multiple	Laboratory only	(Mandel and Higa, 1970, Yoshida and Sato, 2009)
Ultrasonic Gene Transfer	Medium	100µl - 50ml	Medium	Low ($CaCl_2$)	None	Minimal	Wide Potential	Potentially multiple/ natural samples	Potentially in liquid samples	(Song et al., 2007)

1.1.4: Ultrasound, Cavitation and Microbubbles

In the simplest terms ultrasound is an oscillating sound pressure wave with a frequency above the limit of human hearing, generally taken as 20 kHz, and is used up into the gigahertz range (although over 1 MHz is often referred to as 'megasonic'). The reflective properties of these sound waves mean that there are a wide range of uses including echolocation (sonar and mammalian) and imaging (medical scans and crack/fault location in solid materials), the other crucial property is the creation of effect of cavitation generated under certain conditions in liquid. This effect is shown in this work to be the driver behind UGT. This cavitation is an extremely powerful but also extremely localised effect, it can damage steel used in high speed propellers and disintegrate concrete around dam spillways and weirs where hydraulic jumps causing cavitation occur (Berlan and Mason, 1992, Walmsley et al., 1985, Asakura et al., 2008, Thiemann et al., 2011, Wayment and Casadonte, 2002). Cavitation is the formation and subsequent collapse of microbubbles; they are formed where areas of very low pressure are created in a liquid which ruptures to form temporary microscopic voids (Lauterborn and Hentschel, 1985, Lauterborn and Hentschel, 1986). As these microbubbles collapse they create a microjet of high temperature and high pressure (see Figure 4: Microjet formation during Microbubble collapse (Brennen, 1995) which can cause severe damage to solids adjacent to them, however as they are so small they quickly dissipate back to an equilibrium so the net change on a macro scale is insignificant.

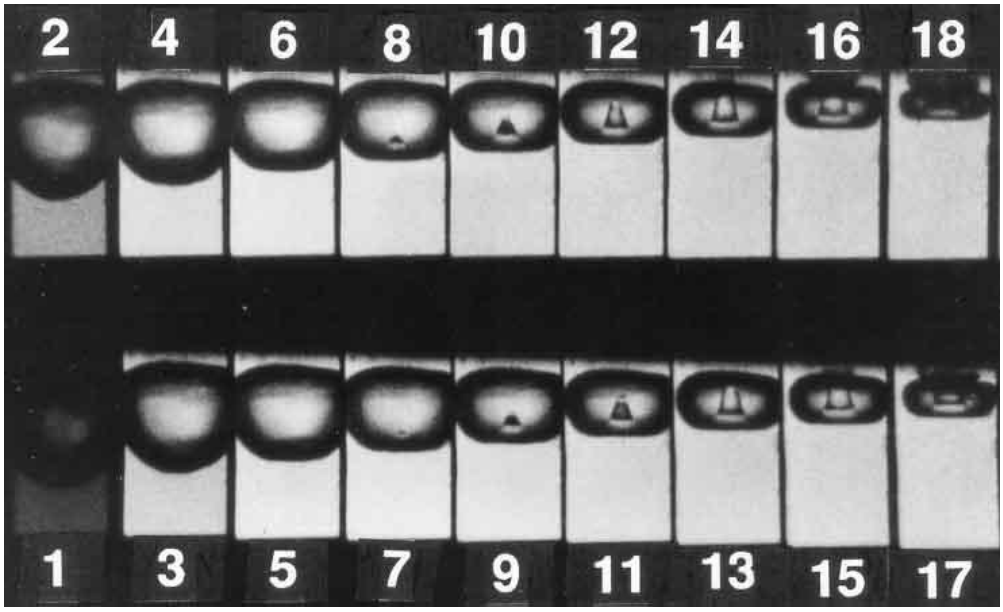

Figure 4: Microjet formation during Microbubble collapse (Brennen, 1995) microjet forms in image 8, penetrates the opposite cavity wall in 12 and extrudes into the surrounding fluid as the remainder of the cavitation bubble collapses

In the case of ultrasonic cavitation: standing waves formed at resonant frequencies of the liquid create pressure maxima and minima, as the liquid passes from high to low pressure it is put under tension. Once the pressure, p, falls below the saturated vapour pressure, p_v, and the tension in the liquid, Δp, exceeds the tensile limit of the liquid, Δp_c, a rupture occurs forming a cavity.

Cavitation occurs when:

$$\Delta p > \Delta p_c$$

Equation 1: Cavitation pressure

Where:

$$\Delta p = p_v - p$$

Equation 2: Pressure differential

The dissolved gases in the liquid and the liquid itself evaporate to form a vapour inside this cavity. This occurs at a roughly constant temperature differentiating it from the boiling effect. Once formed these microbubbles can expand as the pressure allows, conversely as the acoustic cycle continues and high pressure returns the cavity is compressed and begins to critically collapse.

The exact mechanics of collapse are beyond the scope of this thesis but Brennan, C.E. provides an in-depth assessment in 'Cavitation and Bubble Dynamics' (Brennen, 1995), however the resultant effects of collapse are key to the UGT process but temperature should always be monitored as this will directly affect the vapour pressure, p_v, of the liquid and hence the rate of cavitation; whilst each collapse has a miniscule temperature effect the net gain over an extended period can result in heating of the sample and should be controlled to prevent and secondary effects or cell damage (for the standard time periods and volumes of sample used in this work the heating is not of critical importance but nevertheless should be checked).

In a perfect environment the microbubbles would collapse in a spherical manner, however due to irregularities in the surrounding liquid and especially near a liquid/solid interface one area of the bubble will collapse first. This asymmetric collapse creates a re-entrant microjet which rapidly accelerates into the void, it is important to note that this jet is directed toward the interface. The resulting effect of this collapse and microjet formation is an area of localised high temperature, pressure and velocity (in the order of 6700°K, 84.8 MPa (Fujikawa and Akamatsu, 1980)); this creates major engineering problems but can be utilized for beneficial purposes. Such as cavitation being used for ultrasonic cleaning (e.g. jewellery and small mechanical parts) and ultrasonic sterilisation of medical and scientific equipment where it irreparably damages and lyses cells. It is this lysing effect that can be harnessed for UGT where, by limiting the damage, the cell walls can be penetrated without complete lysis occurring.

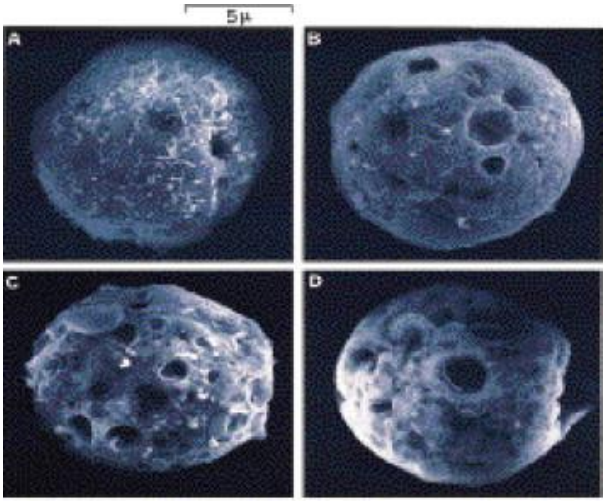

Figure 5: Damage to Mammalian Cell by Sonication from recoverable poration (A & B) to cell lysis (C & D) (Tachibana et al., 1999)

1.1.5: Summary

A new technique for environmental pollution remediation is required. Existing technologies are limited by expense, time, logistics and creating further environmental damage (physical or chemical). Microbiological treatments have the potential to improve this, however adding alien species to indigenous ecosystems is fraught with difficulty and limited by culturable species.

Ultrasound gene transfer can provide the solution. Driven by the cavitation effect it can be performed *in situ*, it is cheap and quick to perform. Unlike other *in situ* techniques the equipment is small and manoeuvrable at an industrial scale removing the obstacle of transport and access to isolated or sensitive areas. The use of indigenous species means the potential for further environmental damage is minimal. There are regulatory hurdles concerning the usage of GMOs in the environment which could restrict the usage to enclosed bioreactor systems; however insertion of single specific degrading genes using plasmids would have no scope for generating untoward cellular changes and once the degradation is complete the genes would no longer confer an advantage and be quickly discarded by natural selection due to the added burden of retaining and reproducing the extra DNA. Furthermore many of the required genes are already in the indigenous gene pool and as such this is a method of horizontal gene transfer simply accelerating natural changes in a manner analogous to crossbreeding plants.

1.2: Aim, Objectives and Thesis Structure

This work will undertake novel research to further the global knowledge pool by investigating a novel technique to transfer DNA into bacteria for the goal of remediation of contaminated land. Specifically, the objectives are:

- To develop the procedure for ultrasound gene transfer in bacteria
- To create, manufacture and adapt an ultrasonic wave generator for *in situ* ultrasonic gene transfer
- To adjust, improve and optimise a procedure for effective *in situ* ultrasonic gene transfer
- To investigate, examine and understand the underlying mechanisms behind ultrasonic gene transfer with respect to micro-bubbles and cavitation
- To assess the transfer of a range of genetic information and macro-molecules into bacteria mediated by ultrasound
- To apply the developed procedure to a range of species in a range of nutrient conditions, including environmental samples
- To insert pollutant degrading genes, specifically salicylate degrading genes, by ultrasound into bacterial species to confer novel genetic traits enabling a previously incapable species to degrade said pollutant

This thesis reports on a cross-disciplinary project covering microbiology, genetics and applications in environmental engineering. By its nature there will be areas beyond the scope of these fields; the aim is to convey the research in a manner accessible to both microbiologists and civil engineers, so readers from both sides can gain an understanding of the potential for ultrasound gene transfer.

This thesis on Ultrasonic Gene Transfer (UGT) is split into three streams of research:

- development of the apparatus
- optimization of the procedure
- application to real world issues

The premise of each will be introduced below and expanded to cover all developments, complications, results, discussion, conclusions and recommendations in the relevant sections.

1.2.1: Development of the Experimental Apparatus

In order to improve and develop UGT from the pioneering investigation by Song et al. (Song et al., 2007) new apparatus was required. Previous work (Boardman, 2008) had used a 40 kHz ultrasonic water bath to transfer plasmids into *Pseudomonas putida* UWC1 and had investigated many optimal conditions for UGT with this apparatus (relative concentrations, temperature, pH etc.) however due to limitations of the single frequency ultrasonic water bath the optimal frequency had not been investigated.

Initial experiments using a variable ultrasonic bath found this unsuitable for rigorous scientific investigation; therefore an ultrasonic probe (sonotrode) was borrowed and investigated. This proved very successful resulting in a robust experimental procedure as a base for further research. Following this success a new probe was designed with improved geometry and materials to increase longevity, generating the final experimental apparatus for the project.

1.2.2: Optimization of the UGT Procedure

Having developed the new apparatus the existing procedure had to be modified from a 1.5 ml flat bottomed glass vial suspended in the water bath. Initially direct contact of the probe to the base of the glass vial was used with experimental conditions similar to that in the bath; this allowed assessment of the optimal transfer frequency from which an insight into the mechanism of transfer was gained.

However in order to develop this technology towards *in situ* use and industrial application simplification and scaling was required. This was achieved by direct immersion of the probe in a cell/plasmid suspension of 20 ml which has significantly more potential for environmental and industrial application. Having investigated frequency, exposure time and relative cell/plasmid/salt concentrations the recommended procedure for UGT has been generated. This procedure is used as the template for all further investigation of applications of the technique.

1.2.3: Areas of Potential Application of UGT

As with any new technology many potential applications have yet to be realised however UGT has the potential to create a paradigm shift in bacterial DNA manipulation by offering the potential ability to transfer DNA *in situ* and into unculturable organisms. High-frequency UGT in mammalian cells (Kim et al., 1996) is a more mature technology from which ideas for applications can be borrowed, including delivery of drugs and large molecules into cells.

This research investigates delivery of a range of plasmids into bacteria including:

- antibiotic markers
- fluorescent markers
- degradation genes for environmental contaminants

These have been delivered into single species in high nutrient conditions; as well as experiments for delivery into low nutrient environmental and industrial samples. Large molecules (up to 2,000,000 MW) have also been delivered to demonstrate potential for drug and macromolecule delivery.

1.2.4: Summary

Whilst the development of the apparatus and procedure was performed concurrently as developments in one led to iterative improvements in the other, this thesis is separated into two distinct streams until the final apparatus and procedure were defined and testing of applications began. Detailed reporting of the development of the apparatus, procedure and experimentation are covered to enable comprehensive understanding of the work undertaken, the complexities involved and the successful transfers achieved.

2: Development of the Apparatus for Ultrasound Gene Transfer

The primary investigation into UGT was performed using a 40 kHz water bath (Song et al., 2007). Whilst other experimental parameters were optimized for this equipment, the optimum frequency for transfer could not be investigated or how a different frequency might affect the transfer process, to achieve this; a variable frequency apparatus is required. The aim of the research in this chapter is to identify the optimal ultrasound frequency for gene transfer into bacteria and to design an alternative variable frequency apparatus for sonication that has the potential for practical use to deliver genes by ultrasound at an industrial scale. This chapter covers the steps used to progress from the single frequency water bath to the second generation variable frequency ultrasonic probe (sonotrode). This section runs concurrently with Section 3 as there was a concurrent development of the procedure whilst the apparatus was developed.

2.1: Variable Frequency Ultrasonic Water Bath

The first new apparatus tested was a variable frequency ultrasonic water bath; on arrival the equipment was uncalibrated for frequency. During the process of calibration it was discovered that the output waveform carried a lot of noise from unwanted frequencies also produced by the apparatus and that the waveforms for each frequency were barely distinguishable, as a result this equipment was deemed unsuitable for rigorous scientific analysis of the optimum frequency of transfer.

To calibrate the variable water bath, a superheterodyne receiver (originally used to differentiate bat sonar which has a similar frequency range) was used; the process behind the receiver is to overlay interference waves so when the incoming frequency is matched no output is generated, i.e. a complementary waveform is generated cancelling the incoming wave, whilst for non-matched frequencies an audible tone is produced as the interference between the two waves being combined generates an output.

However, a clear signal could not be received from the variable bath whilst the standard bath used as a control gave a clear tone at the expected frequencies (see Figure 6), indicating an irregular or wide band frequency being generated by the variable bath as opposed to the single frequency from the standard 40 kHz bath.

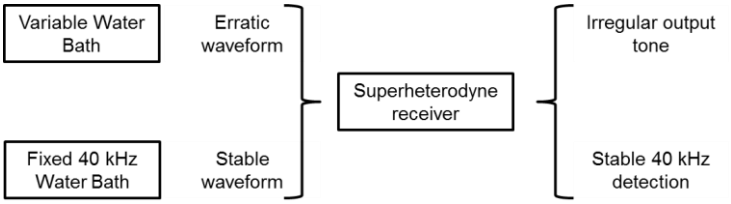

Figure 6: Apparatus for Frequency Analysis using Superheterodyne Receiver

To confirm the cause of the problem an ultrasonic piezo-transducer was connected to an oscilloscope to show the waveform generated (see Figure 7). The standard bath displayed a clean waveform directly corresponding to the expected frequency, however the variable bath produced an erratic base frequency that was overlain by a range of noise and variations of the frequency seemed to have little effect on the wave output. As the frequencies were indistinguishable this apparatus was deemed unsuitable and not used further in this work.

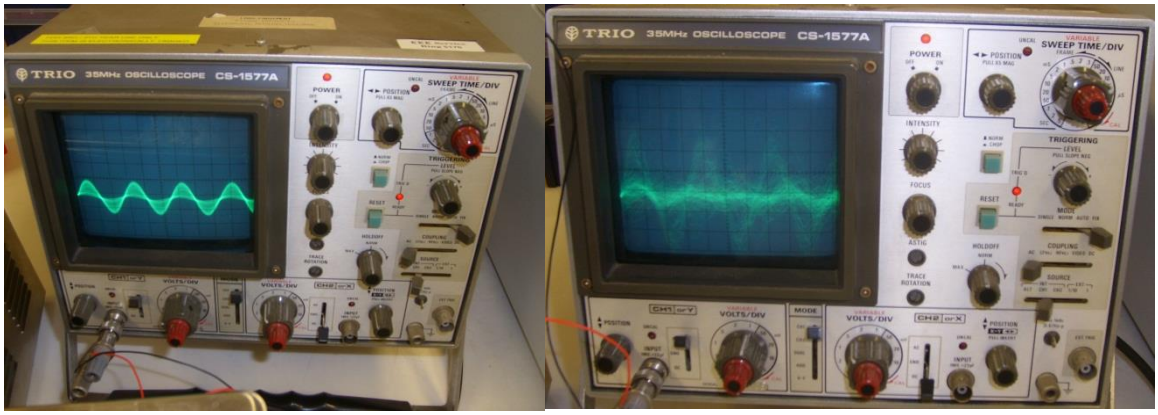

Figure 7: Comparison of Ultrasonic Waveform (Left: Standard Bath, Right: Variable Bath)

2.2: Ultrasonic Probe (model 1)

An aluminium ultrasonic probe (sonotrode) was sourced through collaboration with The Leonardo Tribology Centre, University of Sheffield. The sonotrode was designed and used for crack detection and thin lubricant film analysis however it has a frequency range from low kHz up to over 4 MHz making it ideal to investigate both low frequency UGT as well as assess the possibility of high frequency transfer as well.

Figure 8: Sonotrode Design 1

The apparatus consists of a signal generator (GFG-8255A, GW Instek, USA) used to create the required waveform and frequency linked to a wide range amplifier (75A250, Amplifier Research, USA), the amplifier signal is then passed to a piezo-electric disk mounted in the top of the sonotrode, the signal induces vibrations in the piezo-electric disk which are then directed down the focussing horn to the tip where they are emitted as sound waves (see Figure 8 and Figure 9) (powerultrasonics.com, 1999).

Following initial testing using similar experimental conditions to those used for the water bath, this apparatus was shown to be successful baring some issues with the contact between the tip of the sonotrode and the base of the vial. Neither the vial nor the sonotrode had completely flat surfaces leading to an air gap forming and an unreliable contact between the two. As the ultrasonic waves pass from one material to the next there is an energy loss and loss in quality as the material density changes (from aluminium to air to glass to the cell/plasmid suspension) this is due to partial reflection of the wave energy at the interface. The refraction angle is characterised by Snell's Law which describes the relationships between angles of incidence and refraction passsing through an isotrpoic boundary (e.g. glass to air), using the differing wave velocities due to the density differentials between materials to state:

$$\frac{\sin i_i}{V_i} = \frac{\sin i_r}{V_r} = \frac{\sin i_t}{V_t}$$

Equation 3: Snell's Law

Where:

i_i = angle of incidence

i_r = angle of reflection

i_t = angle of transmission

V_i = velocity of incident wave

V_r = velocity of reflected wave

V_t = velocity of transmitted wave

and the energy losses are characterised by the reflection coefficient (R) which is dependent on the impedence of the material (Z)

$$Z_1 = d_1 * c_1$$

Equation 4: Material impedance (Z)

Where:

d = material density

c = velocity of sound in the material

From which the reflection co-efficient R is calculated:

$$R = \frac{(Z_2 - Z_1)^2}{(Z_2 + Z_1)^2}$$

Equation 5: Reflection coefficient (R)

Where:

Z_1 = impedance in the initial material

Z_2 = impedance in the transmission material

This was reduced by using ultrasonic gel (as used in medical ultrasound scans) to reduced the density step (i.e. d_2 is lower and hence Z_2 thus R is reduced). The gel also improved the contact between the tip and the glass by removing vibration. These loses were eliminated once the procedure was scaled to direct immersion of the sonotrode tip in the cell suspension as there were no longer intermediary phase changes.

This direct immersion setup proved to be initially reliable and robust however the sonotrode was nearing the end of it's operational life as the tip was beginning to degrade as cavitation created during use had caused pitting to occur. This degradation caused overheating both of the sonotrode itself and consequently to the sample as well, this meant a new sonotrode was required.

2.3: Sonotrode (model 2)

Having proven the previous setup as effective for UGT this gave an opportunity to adjust and redesign the apparatus specifically the optimised conditions.

The sourcing and manufacture process was time-consuming taking over a year to obtain the most suitable parts and complete the manufacture to a high standard. Notably the amplifier which was able to amplify in the ultrasonic range was an impediment as most commercial amplifiers are limited to the audible range, resulting in the purchase of a second-hand model from a specialist supplier. Subsequent delays receiving the correct piezo-ceramic disc from the supplier in the USA led to reducing exposure times in an attempt to extend the operational life of the first generation sonotrode.

The function generator used is a standard piece of apparatus and provided the frequency range, accuracy and precision are satisfactory this is interchangeable (model used: GW Instek, GFG 8020H).

The amplifier is more specialised as most commercial acoustic amplifiers only operate up to 20 kHz and often have specific limiters to prevent them amplifying ultrasonic frequencies. As a result ultrasonic amplifiers are much harder to source as they are predominantly used in specialised industrial operations or for military hardware and therefore supply is limited. A few models were identified with suitable specifications, but the most suitable was a minor variation on the model previously used. The model selected was 75A250 made by Amplifier Research (USA), with a range of 10 kHz – 250 MHz and power of 75 W.

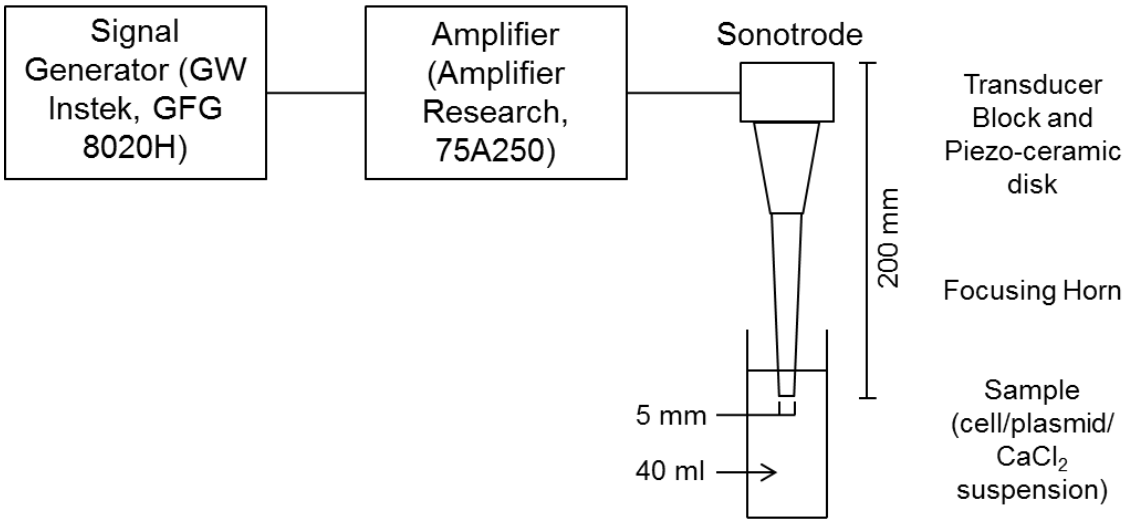

Figure 9: Ultrasonic Gene Transfer apparatus schematic. Sinusoidal waveform generated by GW Instek, GFG 8020H, amplified by solid state 75W broadband RF amplifier, (Amplifier Research 75A250), powering Piezo-ceramic disc, focussed down a two part aluminium and titanium horn

The sonotrode itself was a bespoke construction designed and manufactured in conjunction with the Leonardo Centre for Tribology and the Department of Mechanical Engineering at the University of Sheffield. The design was undertaken by Dr Robin S Mills and manufacture completed by technicians in Mechanical Engineering; the process is outlined in the following sub-sections.

The Sonotrode is comprised of two designed parts: the transducer head containing the piezo-electric ceramic disk and mounting block and a twin section focussing horn (see Figure 10)

2.3.1: Transducer Head Construction

The piezo-ceramic disc was purchased from UESense (USA) with an optimum operating range of 28 kHz (± 1 kHz) at 100W. This was mounted in a cylindrical aluminium block and bolted tightly together to ensure the piezo discs are kept under compression even when the transducer is at maximum oscillation. The ceramic disks have very low tensile strength so even low tensile vibrations can easily lead to cracking and failure of this section of the sonotrode. This also prevents vibrations loosening the mount or damping the ultrasonic waves produced. The vibrations are passed through a high-strength aluminium alloy which completes the retaining transducer head section.

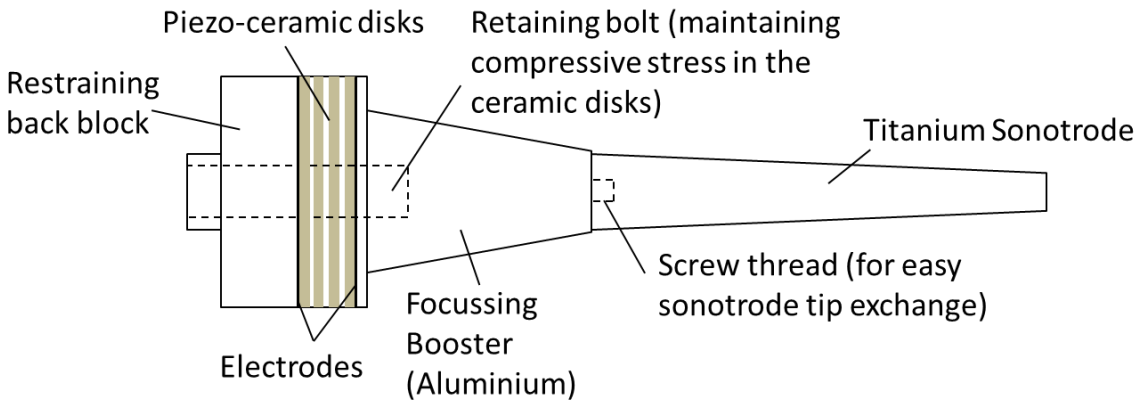

Figure 10: Sonotrode Schematic

2.3.2: Focussing Sonotrode Design

The first section of the focussing horn is a high-strength aluminium alloy with a machined finish and female thread at the end to allow for differing tips to be attached. The geometry was modelled using Matlab computational analysis software. The length is calculated from the formula:

$$l_0 = \frac{c}{2f}\sqrt{1 + \left(\frac{\ln\left(\frac{D_1}{D_2}\right)}{\pi}\right)^2}$$

Equation 6: Sonotrode length

Where:

l_0 = length of section

c = speed of sound in the material (Aluminium = 5100 m/s, Titanium = 4900 m/s)

f = excitation frequency (28 kHz)

D_1 = diameter nearest the source (Section 1 = 45 mm, Section 2 = 20 mm)

D_2 = diameter nearest the tip (Section 1 = 20 mm, Section 2 = 5 mm)

The shape is then generated as an exponential curve for optimum efficiency of energy focussing:

$$\exp(l_0 * u) - 1 = R_1 - R_2$$

Equation 7: Sonotrode shape

Where:

u = unknown exponential multiple

R_1 = initial radius (Section 1 = 12.5 mm, Section 2 = 7.5 mm)

R_2 = final radius (Section 1 = 0 mm, Section 2 = 0 mm)

For machining on a CNC lathe the curve is split 20 mm steps. The radius at each step was found using:

$$y = \exp(u * x) - 1 + R_2$$

Equation 8: Sonotrode stepwise radii

Where:

x = distance from the tip

y = radius of the probe

These points were then joined using circular arcs to approximate the exponential curve to produce a suitable input for the CNC lathe, see Figure 11 & Figure 12.

This shape was designed to minimise losses as the wave front is focussed toward the tip and the length selected to ensure the tip of the sonotrode was at a node and hence produce maximal amplitude for optimum cavitation.

These calculations were performed by Dr Robin S Mills, who then taught the required mathematics and modelling to me. The manufacture was completed by technicians in Mechanical Engineering.

Further information on design and calculation can be found in the ZVEI handbook on Ultrasound (ZVEI., 2006) and at http://www.powerultrasonics.com/ (powerultrasonics.com, 1999).

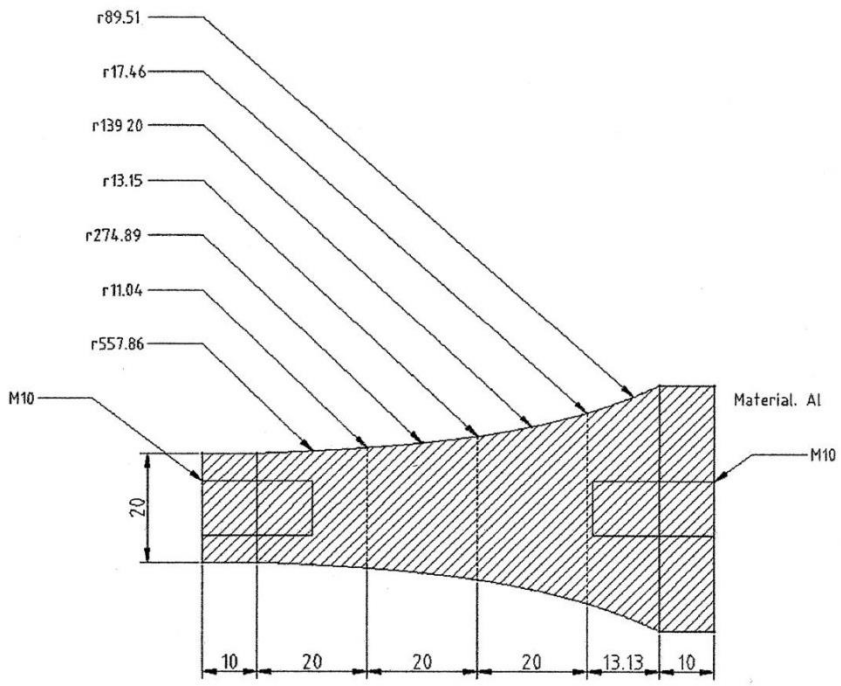

Figure 11: Primary Sonotrode Section (Aluminium)

The second section was similarly modelled and machined from a titanium bar to the dimensions shown below (see Figure 12). Titanium was used to maximise longevity of the tip as the cavitation produced around the tip causes pitting to the surface which reduces the efficiency of producing microbubbles and also leads to rapid overheating of the horn which can damage the samples.

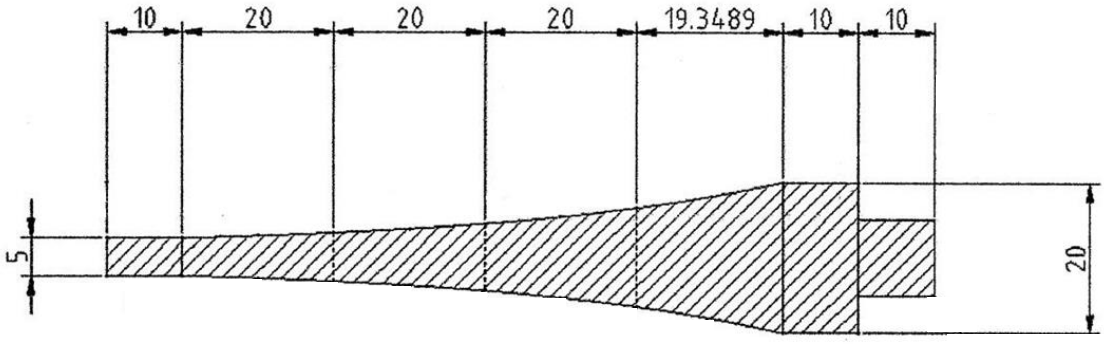

Figure 12: Sonotrode Tip Section (Titanium)

Following manufacture, the sonotrode was commissioned and tested and was found to be similar in output to the previous model and therefore suitable for use for the remainder of the experiments performed in this thesis.

3: Development of the Ultrasonic Gene Transfer Procedure

The development of the operating procedure was at iterative process run in parallel with the development of the apparatus as each change generated new hurdles and optimum specifications.

The base of the procedure was defined by the successful method used by Song et al. in the initial investigation of UGT (Song et al., 2007), i.e. using 400µl of sample for 10 second exposure by a 40 kHz ultrasonic water bath in which a circular kanamycin marker plasmid (pBBR1MCS-2) was transferred into *P. putida* UWC1 and the procedure was optimised for a series of parameters including exposure time and $CaCl_2$ concentration, but notably not for the ultrasonic frequency. This method was also used in my master's dissertation (unpublished data). The master's project was to learn basic laboratory techniques which were then applied to demonstrate repeatability of the previous work of successfully transferring a kanamycin resistance marker plasmid (pBBR1MCS-2). The project then developed the technique transferring green fluorescent protein (GFP) genes on a plasmid into *P. putida* UWC1 (Boardman, 2008) with an efficiency of 10^{-8} and 10^{-9} respectively. Temperature, $CaCl_2$, cell and plasmid concentrations were also investigated creating a baseline for this work.

This section covers the progression from this method to the use of the first generation sonotrode retaining a very similar procedure using glass vials including the use of a gel to improve the transmission of the ultrasound into the sample. Progressing to the direct immersion of the ultrasonic probe into larger volumes of sample; this is the first time DNA transfer in bacteria has been performed in this manner. This is aimed toward progression to *in situ* application for environmental samples.

For the first time a comprehensive range of frequencies (15 kHz – 3 MHz) has been investigated and exposure times assessed to explore optimum conditions for UGT, as well as varying concentrations of the cell/plasmid suspension and other potential mediating chemicals.

The direct immersion procedure is currently the recommended final method and baring minor adjustments for specific experimental requirements was used to test

UGT across a range of samples and to investigate the mechanism behind UGT, throughout this work.

3.1: Sonotrode, Glass Vials and Ultrasound Gel

Abstract

Preliminary investigation into the properties and effectiveness of the ultrasonic probe has shown that it is a successful method of delivering ultrasound for UGT. A range of ultrasound frequencies were tested to compare the efficiency of transfer of the probe with that of the ultrasonic water bath and to investigate the optimum frequency for ultrasonic gene delivery. Transfers were performed with samples prepared using the method previously described by Song et al. (Song et al., 2007) with the base of the vial placed directly on the tip on the sonotrode and a range of frequencies were used. Peak transformant count were recorded at 27.6, 40 and 50 kHz with two further peaks at 600 and 800 kHz; these frequencies corresponded to frequencies at which high levels of agitation of the sample was noted during sonication. Transfer efficiency was improved by the application of a layer of ultrasonic gel between the tip of the sonotrode and glass vial to reduce energy loses; this improved transfer rates at 600 kHz but not at lower frequencies. This data confirms the success of the ultrasonic horn at a comparable transfer rate to that of the bath, thus providing a base point for further investigation. Further progression into optimising the transfer frequency and improvement of other aspects of the procedure towards *in situ* application will require scaling and the use of direct immersion of the sonotrode in the sample is proposed.

Introduction

These are the first stages of developing the procedure providing the step from the ultrasonic bath to the sonotrode and hence to analysing the optimum frequency of this equipment. Having found the variable ultrasonic water bath to be unsatisfactory; collaboration with The Leonardo Tribology Centre in the Department of Mechanical Engineering at the University of Sheffield was developed. A fruitful partnership with Chris Rose, who was using the apparatus for oil film measurements, was formed allowing access to the equipment and expertise as to its operation.

The primary aim of this initial investigation is to demonstrate that plasmid DNA can be transferred into *P. putida* UWC1 using an ultrasonic sonotrode at a comparable

efficiency to previous work using a 40 kHz ultrasonic water bath. A secondary aim is to gain an indication as to the optimum frequency of transfer and any insights into the mechanism of transfer.

This is followed by investigating methods to improve the glass vial procedure, notably the contact between the tip of the sonotrode and the glass vial. Currently the ultrasonic waves pass from the titanium tip of the sonotrode through an air gap at the concave base of the glass vial being held in place by hand, and then into the liquid media. This involves 3 phase changes of material including solid to air and back again, at each stage the transmitted energy in the wave is lots due to absorbance and reflection; the larger the density differential the more energy is lost. In an attempt to improve this some off-the-shelf **ultrasonic gel** used for medical scans was tested with the hypothesis that this should act in a similar manner as in medical usage. There is also expected to be a reduction the vibration loses generated holding the vial in place.

For full details of the sonotrode apparatus see Section 2.2: Ultrasonic Probe (model 1).

Materials and Method

Ultrasonic transfer experiments used the plasmid pBBR1MCS2 as the transferred genetic information, containing a Kanamycin resistant gene to act as a marker and *Pseudomonas putida* UWC1 as the recipient strain. The plasmid was grown in *Escherichia coli* K12 DH5α and prepared using a Qiagen Miniprep kit (QIAGEN, UK) as per the manufacturer's protocol. 20 ml per sample of *P. putida* was grown overnight at 27 °C in LB broth medium and then pelleted by centrifugation (3 min at 3000 g) and re-suspended in 200 µl of supernatant. 200 µl of 100mM $CaCl_2$ was added in 1.75 ml flat bottomed glass vials (Richardsons of Leicester Ltd., UK) and 10 µl of plasmid (5 ng/µl, suspended in dH_2O) was added. This was exposed to 10 s of ultrasound at frequencies of 25, 27.6, 30, 35, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 3000 kHz; for each frequency 5 replicates were performed.

For the **ultrasonic gel** comparison the cells were prepared as above. Prior to sonication a layer of ultrasonic gel (Anagel, UK) was spread between the sonotrode tip and the base of the vial. Samples were sonicated for 10 s at 40 and 600 kHz with 4 replicates with and without the gel.

All work was performed at room temperature (21°C) unless otherwise stated. Samples were then incubated for 2 h at 27°C to allow for recovery then 100 µl of sample was spread on LB agar plates with 50 µg/ml Kanamycin supplement for transformant selection. Samples were also diluted to $x10^{-6}$ and spread on LB agar plates with no selective pressure to assess cell survival. Resultant colonies were counted after overnight growth. The difference between groups was analysed by one-way ANOVA using SPSS (IBM, USA) which compares the means of the groups against the variance of the sample to give a probability whether the groups are distinct sets or the difference is not statistically significant.

Results

The initial results using the sonotrode across a range of ultrasonic frequencies in Figure 13 show a wide variation in CFUs produced, from negligible change from control samples to marked increases in number of transformants.

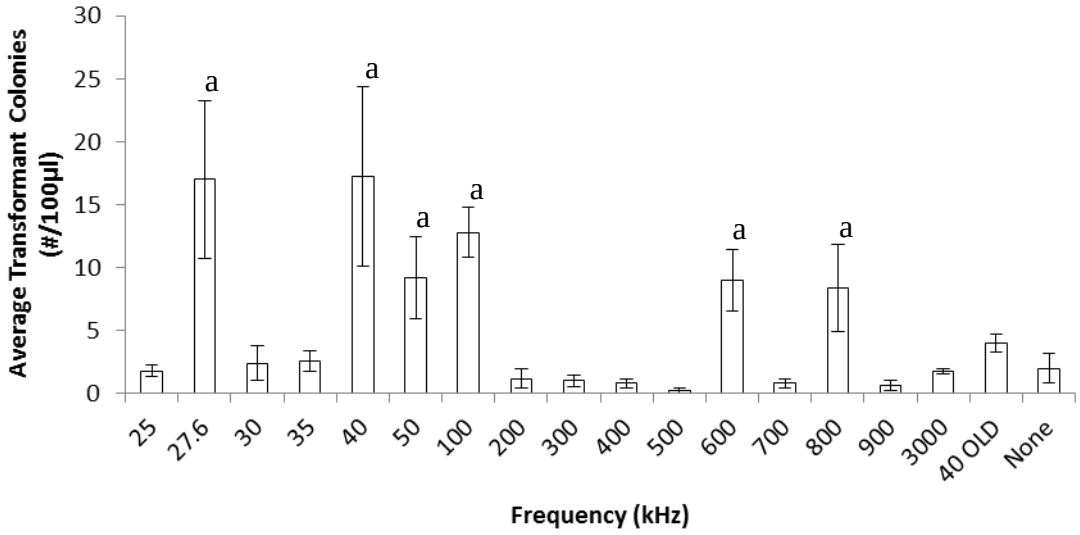

Figure 13: Number of transformants for a range of ultrasonic frequencies 10 s exposures of 400 µl sample of *P. Putida* UWC1 and 10 µl of plasmid pBBR1MCS2 in 50 mM CaCl2, mean values of five biological replicates, error bars of 1 S.E.

Increased transfer rates occur at 27.6, 40, 50, 100 kHz as well as two further peaks at 600 and 800 kHz, the other frequencies show no noticeable improvement over transformation without ultrasound. During exposure it was also noted, that for the frequencies noted above (marked ‘a’ in Figure 13), agitation and turbulence was seen in the sample but not noticeable in the unmarked others.

It should be noted that natural transformation without ultrasound or otherwise mediated has not previously been reported in this strain (Johnsborg et al., 2007) therefore the transformants on the controls without ultrasound were unexpected. Whilst the number transformed is low compared to the samples with ultrasound, the samples with no plasmid produced no growth on any of the selective replicates showing no natural antibiotic resistance. This is a novel finding that *P. putida* UWC1 can naturally uptake plasmid DNA.

Analysis by One-way ANOVA shows that the data sets are statistically different ($p =< 0.001$), followed by a pairwise multiple comparison using the Student-Newman-Keuls Method, which showed the higher transfer rates (marked 'a' in Figure 13) to be significantly different from the baseline level of the remainder and the negative control of no ultrasonic exposure ($p<0.05$), the remainder were not shown to be significantly different from the small data sets used in this experiment.

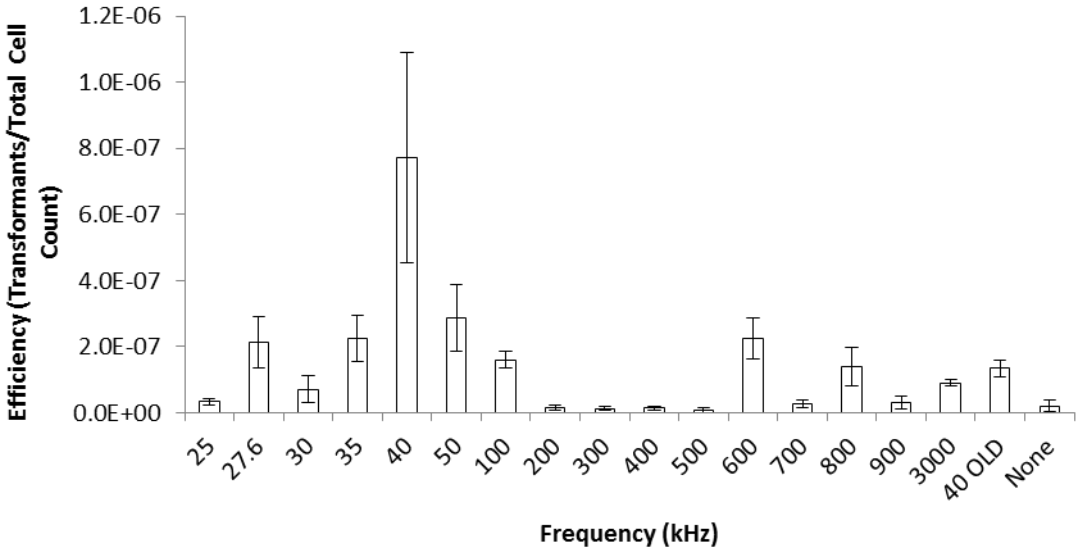

Figure 14: Transfer Efficiency for each Frequency mean number of transformants on selective media (see figure 1) divided by the total cell count on non-selective media

When compared using the efficiency of transfer by calculating the ratio of transformants against total cell survival rates (colonies formed on non-selective plates) a similar trend is also seen; however higher rates of cell death at higher frequencies led to increased transfer efficiencies for >100 kHz samples as the relative cell count following exposure is lower (200-300 x 10^4 CFU/ml), especially at

600 and 800 kHz, whilst at 27.6 kHz cell survival was comparable to the unexposed samples (both ~ 600 x 10^4 CFU/ml).

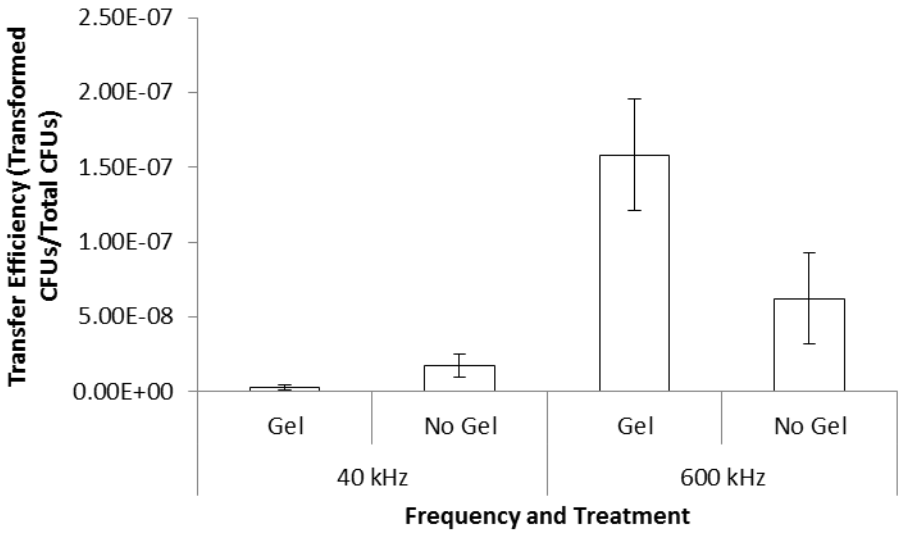

Figure 15: Effect of Improving Ultrasound Transmission with Ultrasound Gel average efficiency of 4 replicates with 1 S.E. error bars (*) is significantly different

When comparing samples with the **ultrasonic gel** treatment between the vial and sonotrode tip: at 600 kHz a statistically significant increased transfer rate was seen with a one tailed t-test of p_0 <0.05 that the null hypothesis that there was no difference between the gel and no gel samples was true; whilst the test for 40 kHz a drop in transfer was seen and the null hypothesis that there was no increase in transfer rate with gel at this frequency was accepted as p_0 >0.05.

It was also noted that visually it was observed that for the gel tests that the cell/plasmid suspension was more agitated, the difference was however less noticeable for 40 kHz than 600 kHz.

Discussion

It can be seen by the results that the probe is a successful alternative to the water bath and a range of frequencies are suitable of mediate plasmid transfer into the recipient bacterial cells. During the exposure agitation of the sample could be clearly seen around the resonant frequencies which also corresponded to increased transfer efficiency, this is consistent with the theory that the transfer is mediated by the collapse of micro-bubbles generated by the ultrasound. The lower frequency peaks

match the resonant frequency of the sonotrode and subsequent harmonic frequencies, the higher frequencies are harder to explain however it is suggested that a resonant frequency due to the internal reflection from the specific geometry of the vial is the cause as different sized vials have not shown agitation at these frequencies.

When assessing the efficiency the rate of cell death should be monitored and the application for which the transfer is to be used should be taken into consideration to select the appropriate frequency, as the most damaging frequencies resulted in nearly 50% cell death, however this will be further investigating when optimising exposure time. If the number of transformants is key a high frequency with resonance is suitable, but for an *in situ* sample where it is important not to damage too many cells a lower resonant frequency may be preferable.

The effects of the sonotrode are similar to those found using the ultrasonic bath with similar numbers of CFUs remaining following sonication under comparable conditions. Even for the highest rate of cell lysis the cell survival is still markedly higher than found in electroporation. The damage to the cells is not identified and the use of a high speed microscopic camera would give an exact understanding of the processes occurring, as well as images of the cell morphology before and after sonication to investigate the physical effects of sonication on the cells.

It is shown that the **ultrasonic gel** improves transfer efficiency at 600 kHz and hence the gel is effective at improving ultrasonic wave transmission at this frequency however at 40 kHz no improvement is seen. Considering ultrasonic gel is designed for use in the MHz range this is not unexpected. The net benefit of the gel across the frequencies is not seen as sufficient with respect to the relative complexity of application and considering the goal of a simple procedure the advantages are not adequate for continued development in this direction.

The increased agitation of the samples when a resonant frequency is applied where the cell suspension is seen to circulate within the vial, shows increased transmission of energy into the samples however with the variation between the tested frequencies the effectiveness of this is uncertain and suggests that another factor in the transfer mechanism may be involved beyond only improvements to the sample mixing.

Conclusions and Progression

For maximum number of transformants the lower cavitation frequencies are best (27.6 and 40 kHz) whereas for efficiency the resonant frequencies at 600 and 800 kHz are better. There is also a correspondence between high transfer efficiency and disturbance of the sample where circulation and agitation driven by the sonication can be observed; this could provide a key to the transfer mechanism however this was not expressly investigated here and warrants scrutiny to understand the interactions between cells, plasmids and the ultrasound waves to investigate the theory that cavitation is the driving mechanism behind low frequency ultrasonic gene transfer.

The use of **ultrasonic gel** improves transfer at 600 kHz but not at 40 kHz, this lack of conformity between the frequencies combined with the added burden of applying the gel reduces the usefulness of this result and is hence not recommended except for specialised small scale transfers.

Further investigation into the optimum frequency of transfer and adjustments to the procedure to increase efficiency are required, including improving the transmission of the ultrasonic waves from the horn into the cell/plasmid suspension. Considering the aim of the developments is towards an *in situ* application, this line of experimentation is leading toward a dead end with respect to the overall research goal, thus further work with the vials is not recommended and progression to completely remove the phase changes and related loses by direct immersion of the sonotrode in the cell/plasmid suspension is suggested, over alternate methods to increase efficiency with the glass vials.

3.2: Direct Immersion of the Sonotrode into the Cell/Plasmid Suspension

Having reached a practical limit on improvements to the glass vial the biggest step for improvement was to reduce the energy losses due to reflection of the sound waves as they travelled from the sonotrode through the air/gel then glass before reached the sample, to achieve this direct immersion of the probe tip was proposed. This also allowed for scaling of the procedure to a millilitre volume level. This enabled visual observation of microbubbles forming and collapsing and hence the hypothesis and testing that cavitation is the driver behind UGT.

3.2.1: Comparison of Efficiencies

Abstract

Direct immersion of the sonotrode provides a comprehensive jump in simplification of the UGT procedure, however there is an overall loss in transfer efficiency relative to the glass vial with gel transfers, but parity with respect to transfer efficiency using the 40 kHz water bath. A base point for optimising the procedural parameters has been formed and a hypothesis that the resonant frequencies in the cell/plasmid suspension are key to transformation.

Introduction

The ultrasonic horn has been shown to be successful at transferring plasmid DNA into bacteria; however the use of glass vials is restrictive and requires a series of steps to manipulate and concentrate the cells. The aim of this technology is to be quick and simple compared with other competing methods, as such direct application of the horn into the cell suspension is suggested as an effective simplification. The direct immersion method both removes the need to concentrate and transfer the cells between vessels prior to sonication and it also removes the phase changes and resultant energy loses, from reflection and absorption, for the ultrasonic waves from horn to air to glass to cell suspension and associated energy loses.

However with direct contact between the sonotrode tip and the cell suspension steps to avoid contamination of the samples will need to be implemented. Practical alterations made to the apparatus and method for ease of application including increasing the volume of cell suspension so the tip of the sonotrode will fit in the sample vessel and be adequately submerged to avoid surface disruption or inconsistency in the sonication.

Materials and Method

Overnight growth (starter colony inoculated in LB broth incubated overnight (~16 h) at 27 °C) of *P. putida* UWC1 is decanted into 20 ml samples and 20 ml of 100 mM $CaCl_2$ is added. Plasmid pBBR1MSC-2 (grown in *Escherichia coli* K12 DH5α and prepared using a Qiagen Miniprep kit (QIAGEN, UK) as per the manufacturer's protocol) was added in the selected volumes (50 µl and 100 µl) and mixed. The tip of the probe was then immersed in the suspension and ultrasound applied for 10 s at the chosen frequencies (27.5 and 600 kHz). This is followed by 2 h incubation for

cell recovery and gene expression, 100 µl was spread on selective agar plates containing 50 µg/ml of Kanamycin to estimate transformation efficiency relative to non-selective control plates.

Positive controls using the old 40 kHz water bath and original procedure were used as well as testing using the glass vials and ultrasound gel (see Section 3.1.2).

Results

Initial results showed no growth on selective plates for the direct immersion samples however following concentration (x 40) and re-plating, colonies were seen on the plates. Efficiency was calculated from the average number of transformed CFUs on Km_{50} selective agar plates over the total CFUs on non-selective agar plates.

Table 2: Direct Immersion Results

Frequency (kHz)	**Plasmid Volume (µl)**	**Efficiency** (transformants/total CFUs on non-selective agar)
Direct Immersion		
600	50	5.0E-09
600	100	1.3E-09
27.5	50	8.7E-08
Glass Vial and Gel		
600	10	5.5E-07
27.5	10	2.9E-08
Old Bath		
40	10	1.1E-08

During these exposures it was noted that at 600 kHz lower agitation of the sample was seen in the direct immersion method relative to 27.5 kHz and in the glass vial and also has the lowest transformation efficiency. The glass vial tests produced the highest transformation at 600 kHz however the direct immersion was markedly lower.

In the 27.5 kHz samples close inspection identified micro-bubbles being produced at the tip of the sonotrode, dispersing and then collapsing in the cell suspension. It is expected that the turbulence in the glass vials is also due to this mechanism but the opacity of the high cell concentration and small sample obscured investigation.

Discussion

The noted differential in efficiency between the set of ultrasound frequencies in which agitation of the sample was seen versus those in which it was not suggests that this is a key aspect in the transformation method. Similarly the rate of formation and collapse of micro-bubbles at different frequencies and thus its impacts of transformation efficiency warrant deeper investigation, it is suggested that the natural resonant frequency of the cell suspension and the respective harmonic frequencies and the resultant cavitation produced could play a central role in this.

This however fails to explain the higher transformation seen in the vials at 600 kHz. Following discussion with Chris Rose, the collaborator for the sonotrode equipment, it was reasoned that this could be caused by reflections of the ultrasonic waves within the glass vials creating superposition of the waves and hence why this is not seen in the larger and differently shaped 40 ml direct immersion samples. As the focus shifts towards the direct immersion sample further confirmation of this was not undertaken.

Whilst the transfer efficiency for the direct immersion samples may be lower this is not entirely unexpected; the direct immersion sample has a hundredfold lower concentration of cells and a twentyfold lower concentration of plasmids. These lower concentrations will results in a decrease in interactions between the cells and plasmids and the ultrasonic waves. This should however be mitigated by the reduction in energy loses due to phase changes without the glass vial.

It is expected that the transformation efficiency can be increased for direct immersion by optimisation of the procedure for: frequency, exposure time, cell/plasmid concentration, mixing/stirring of the sample, calcium concentration effect and differences in the cells' growth phase.

Conclusions and Progression

Direct immersion has been proven as a successful method to mediate gene transfer and has high potential for efficiency increases; focussed around the resonant frequency found in the cell/plasmid suspension as well as increased exposure time and investigation of other experimental parameters.

Despite the decrease in transformation rate the increase in experimental simplicity is clear and provides an excellent base to develop the procedure toward the goal of *in situ* application.

Efficiency should be improved by changes to the procedure, however this is not the fundamental goal as natural selective pressures should ensure increased reproduction of the transformants *in situ* and improvements to the community once genes are introduced and confer an advantage to the recipients, as such a high percentage of cells being transformed is preferable but not vital.

3.2.2: Optimisation of Ultrasonic Frequency on the Transfer Rate and Investigation into whether Cavitation is the Mechanism behind UGT

Abstract

Following testing over a range of ultrasonic frequencies, UGT by direct immersion of the sonotrode has been shown to solely occur at the resonant frequencies where micro-bubble formation and collapse takes place. The cavitation effect damages the cells potentially allowing the plasmid DNA to enter; however over exposure lyses the cells; optimising the balance of this effect is the key to developing this procedure further. Close investigation has led to cavitation being recognised as the driver behind UGT, with cavitation being shown as the driver by testing frequencies +/- 1 kHz either side of the cavitation frequency. At resonant frequency micro-bubbles are generated and collapse driving UGT whilst frequencies less than 1 kHz away lead to no transfer. The optimum frequency for this apparatus of ~27.5 kHz is recommended for future work; dependant on experimental variations. Overcoming the difficulties of real time imaging of this mechanism would be invaluable in understanding the micro-scale effects however this was not achieved within this project.

Introduction

Having shown direct immersion of the sonotrode in the cell/plasmid suspension is a viable method for UGT the next stages are to optimise the procedure and to develop an understanding of the mechanism behind the transformation.

A range of frequencies were tested to determine the optimum for UGT. Following previous work which suggested resonance and the related micro-bubble formation and collapse had an effect on transfer efficiency, there is building evidence from comparison between transfer rates for cavitation frequencies v non-cavitation, to suggest that these cavitation forces from micro-bubble collapse are the driver behind UGT. Following a review of progress it was felt that the frequency data was not sufficiently conclusive to demonstrate that cavitation was the driver rather than caused by the ultrasonic wave itself and a further experiment between resonant and non-resonant frequencies was needed to definitively show this. Therefore the hypothesis is that the resonant frequencies (~15.7, ~27.5 and ~40 kHz) will result in transformation whilst the frequencies ± 1 kHz will not mediate transfer.

(Note: this procedure was performed after the time analyses so some of the experimental conditions have been changed from the surrounding experiments however it is more logical to group it here with the frequency analysis data.)

Materials and Method

Following the procedure developed for the direct immersion tests; overnight growth of *P. putida* UWC1 in LB broth is decanted into 20 ml samples and 20 ml of 100 mM $CaCl_2$ is added. 50 µl of extracted plasmids are added and the mixture is exposed to ultrasound for ten seconds by immersion of the probe tip in the sample at the selected frequencies: 15, 15.7, 20, 25, 27.5, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 3000 kHz with 5 replicates and for the specific cavitation frequency tests 27, 27.3 (resonant cavitation frequency) and 28 kHz. Positive controls using the water bath and negative controls with no ultrasound and no plasmids were performed in triplicate. All samples are then incubated for two hours at 27 °C following sonication for cell recovery and gene expression as is commonly performed in other transfer technologies prior to plating on selective agar plates.

(Note: The exact frequency may vary slightly due to experimental and environmental conditions affecting the sample and the sonotrode and should be checked for maximal cavitation prior to each experiment.)

Results

The range of frequencies tested by direct immersion of the sonotrode in the sample only provided only a few transformation frequencies and at low transfer levels however these directly correlated with the resonant frequencies of 15.7, 27.5, 40 and 50 kHz. No transformation or micro-bubble formation was seen at other frequencies.

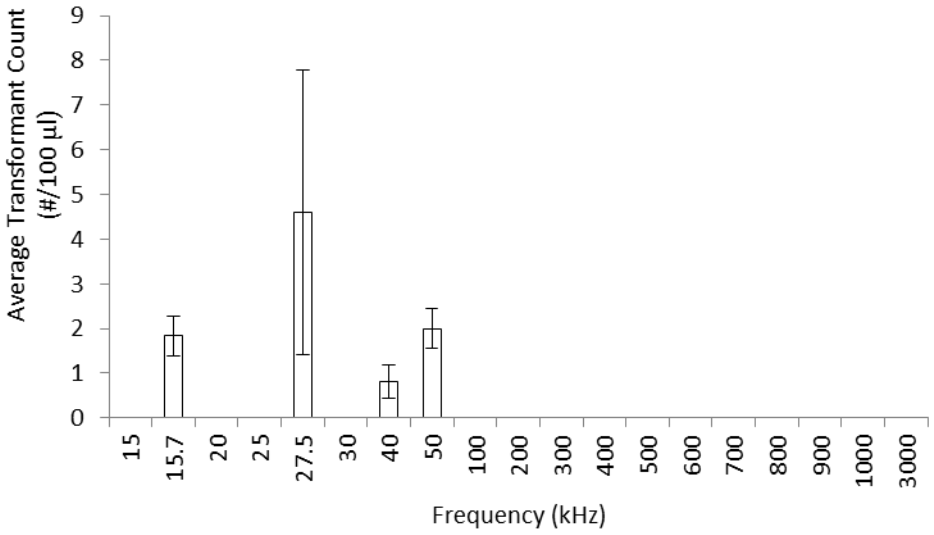

Figure 16: Transformant Count with Change in Frequency by Direct Immersion average of 5 replicates with 1 S.E. error bars

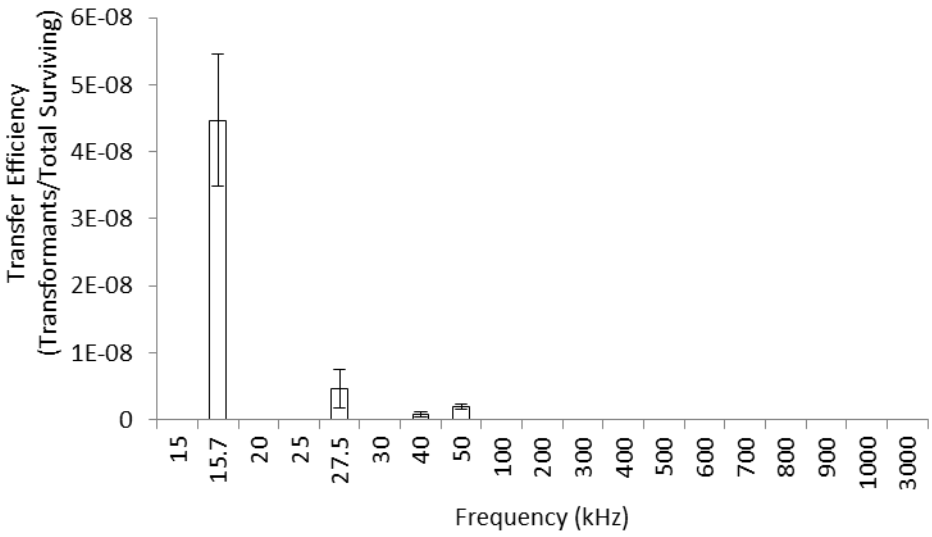

Figure 17: Transfer Efficiency by Direct Immersion average of 5 replicates with 1 S.E. error bars

Given the few numbers of transformants the efficiency is a less valid comparison here however where no micro-bubbles formed/collapsed the cell survival was similar to that of no exposure to ultrasound, whilst for the lowest resonant frequency of 15.7 kHz there was a cell survival of just 10% and the effects visually observed in the sample during sonication were very aggressive. This resulted in a markedly higher efficiency (see Figure 17).

The positive controls using the old water bath gave high transformant counts of ~200, a hundredfold higher than for any of the probe frequencies.

When focussed directly about the cavitation frequency a marked differential of transformation counts is shown:

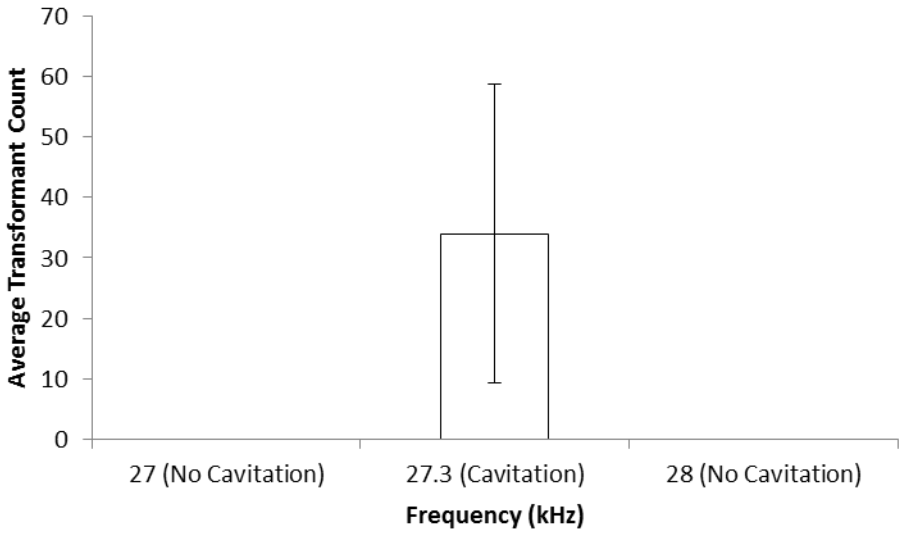

Figure 18: Transformation at Cavitation vs. Non-cavitation Frequencies
Average of 5 biological replicates with 1 S.E. error bars

The data shows no transformants at 27 or 28 kHz however at 27.3 kHz where cavitation was seen transformation occurs in each of the five biological replicates. No efficiency was calculated as this was a direct comparison as to whether UGT occurred or not in each sample.

Following an ANOVA the groups were statistically significantly different ($p=0.001$), the following pairwise Tukey test shows with $p<0.05$ that 27.3 kHz is significantly different from both 27 and 28 kHz.

Discussion

These results show a strong relationship between the occurrences of resonance induced micro-bubbles and gene transfer. The lack of transformants at higher frequencies corresponds to the visual inspection of the sample during exposure where turbulence from sonication could not be seen. When this relationship is further investigated the dependence of transformation on cavitation is confirmed.

The increased cell death rate at the lower frequencies suggests irreparable damage is being done to the cells as the energy transfer from cavitation is too high. 27.5 kHz produces fewer cell deaths and a higher average number of transformants and thus is suggested as the preferred frequency for future investigation. Whilst the transformant count is much higher for the ultrasonic bath over the sonotrode the reduced cell

concentration and increased simplicity in the sonotrode procedure still preserves the potential for progression towards *in situ* applications.

When the focus was on the cavitation frequency the data conclusively shows that cavitation is the driver behind UGT as transfer only occurs where cavitation is seen and if the frequency is even slightly off the resonant frequency then no cavitation and hence no transformation is seen.

A wide variation between samples at 27.3 kHz is noted a and it would be useful to understand why with one replicate produced a markedly higher number of transformant colonies when grow on the selective media; whether this is due to sampling variations due to poor mixing when sampling to place on the agar plate, a slight difference during experimentation or just biological variation, the ability to harness this high rate would beneficial.

(Note: Details on research into the micro-bubbles and their collapse, known as cavitation is detailed in Section 1.1.4. Further investigation is also undertaken in Section 3.3.1 whilst testing the second generation sonotrode and confirms the precision of the resonant frequency effect.)

Conclusions and Progression

Cavitation has been shown to be the driver behind UGT; with the resonant sonication frequencies clearly agitating the cell/plasmid suspension and micro-bubbles being formed and then collapsing. Cavitation damages the cells to the point of destruction in some cases therefore a balance of the ultrasonic frequency and exposure time needs to be found to give a Goldilocks point where sufficient transformation occurs but without fully lysing the cell and preventing cell wall repair. The resonant frequency at ~27.5 kHz meets these criteria and hence exposure time is the next parameter to be investigated for increasing efficiency. Ideally imaging the micro-bubble collapse and subsequent damage to the cell wall, which is hypothesised to be the mechanism of transfer of the plasmid into the cell as opposed to an alternative effect of the free radicals also produced during cavitation or another unknown mechanism. However a camera capable of suitable resolution, speed and ability to cope with the vibrations generated by the sonication was not sourced during this work.

3.2.3: Optimisation of Plasmid Concentration and Sampling Mixing on Transformation Efficiency

Abstract

Two variations in the procedure are investigated: an increase the volume of plasmid added to the cell suspension prior to sonication and the use of a vortex to mix the sample during differing ultrasonic exposure periods. A direct relationship between the total mass of plasmid added and transfer efficiency was identified, however a peak rate was not achieved in the range of this experiment. The largest plasmid mass that is practical to extract is recommended for use. By increasing ultrasound exposure times more transformants were produced and the effect of mixing during exposure on transfer efficiency was found to negligible with respect to the added complexity of experimentation. Mixing did, however, reduce the variance between samples. Given ultrasonic exposure time has previously been optimised for the ultrasonic water bath at 10 s, a change of apparatus and experimental conditions, including ultrasound frequency and cell mixture concentrations, the optimal time for exposure was re-assessed. Investigation up to 1 min showed an increase in transfer rates; hence an increase in exposure time gives an increase in transformation, however a peak has not been reached, so a wider range of exposure times must be investigated. Adverse effects of longer exposures causing cell lysis must also be considered during this process.

Introduction

Having demonstrated the direct immersion procedure successfully mediates UGT; improvements to transfer efficiency were targeted. The concentration of plasmid in the cell/plasmid suspension to be sonicated was investigated as the increase in sample volume is expected to have a different optimum plasmid concentration. It is hypothesised that there will be an approximately linear relationship between plasmid concentration and transformation rate, up to a maximum level, it is uncertain whether that maximum will be reached within this experimental limitations.

The exposure time will also be investigated for the chosen 27.5 kHz frequency; the water bath had been optimised with 10 s exposures and it is reasonable to assume that for differing equipment and a much larger sample volume the optimum exposure time will be longer. Increased exposure will lead to more cavitation/cell/plasmid

interactions and therefore expected to increased transfer rates. However excessive expose will lead to irreparable cell wall damage and lyse the cells, which is not desirable. It is also a concern that with an increased exposure time that the cells near the tip of the sonotrode will be irreparably damaged by cavitation whilst those further away will not be sonicated at all so agitation of the sample has been proposed to mitigate this.

Materials and Method

The direct immersion procedure was followed; using an exposure frequency of 27.5 kHz and 20 ml of overnight growth supplemented with 20 ml of 100 mM $CaCl_{2.}$ For assessment of the effect of plasmid concentration, Maxiprep (QIAGEN, UK) plasmid extracts (~5 ng/µl concentration, quantified by agarose gel electrophoresis, see Figure 19) were added in volumes of 10, 50 and 100 µl (5 replicates), for a final concentration of 1.25, 6.25 and 12.5 ng/ml of cell suspension respectively, compared to a concentration of 125 ng/ml in the 40 kHz waterbath procedure. The samples were sonicated for 10 s each. For investigation into time and mixing effects 100 µl of plasmid DNA (~5 ng/µl) was added, the samples were exposed to 27.6 kHz for 10, 20, 30 and 60 s, during ultrasound exposure the samples were agitation continuously using a bench-top vortex mixer at 600 rpm or held statically, 3 replicates of each treatment were performed. Negative controls with no ultrasound or no plasmid were also performed. Following the low transformation rate seen in previous experimentation the samples will be concentrated 100 times by centrifugation prior to spreading on selective agar plates.

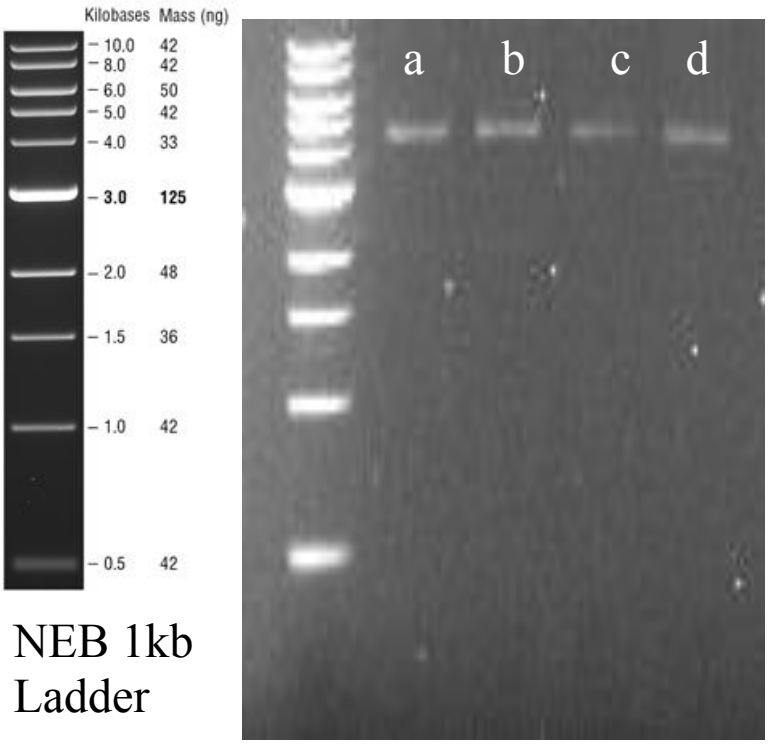

Figure 19: Agarose Gel of Plasmid pBBR1MCS-2 (a-d) extracted by QIAGEN Maxiprep, with 1kb ladder (New England Biolabs)

Results

Plasmid Volume: There is a direct correlation between the volume added and the number of transformants however the rate increase is sharper than expected, which suggests a sigmoidal relationship with a levelling off once plasmid concentration is no longer the limiting factor. However no peak is reached within this range, due to the practical limitations of plasmid production facilities prevent higher volumes being used and hence higher plasmid concentration.

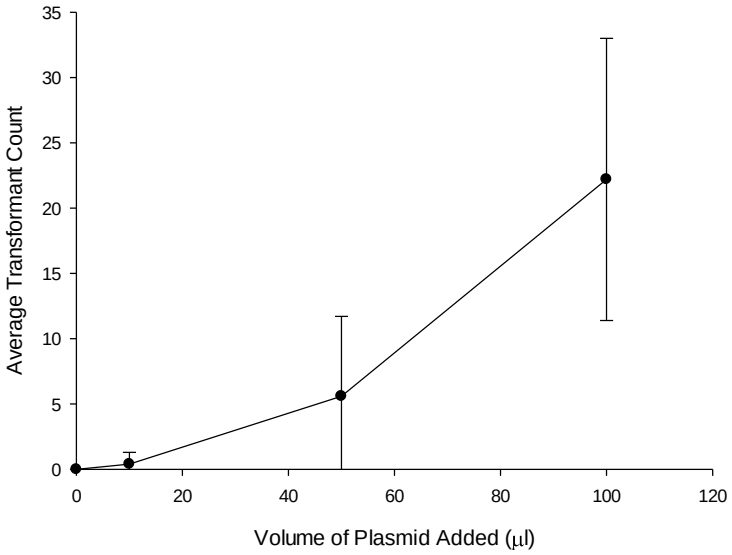

Figure 20: Change in Transfer Rate with Increased Volume of Plasmid Added
Average values of 5 replicates with 1 S.D. error bars

The controls with no ultrasound and 100 µl of plasmid produced a few transformants (0-5 CFUs) showing some natural transformation but at an average rate 20-fold lower than that found using ultrasound.

The positive control using the ultrasonic bath (plasmid concentration 125 ng/ml) gives an average number of transformants (average = 19 colonies) a hundredfold more than in the 40 ml sample with 12.5 ng/ml plasmid concentration (average = 22.2 colonies, concentrated 100 times). This differential with a tenfold increase of plasmid volume in the positive control supports the hypothesis that UGT is strongly dependant on the plasmid concentration.

Mixing and Initial Exposure Time: The data shows an upward trend of transformants with increased exposure time (see Figure 21), there is a large variance of the replicates for each exposure time and only the 60 s exposure is a statistically significant effect.

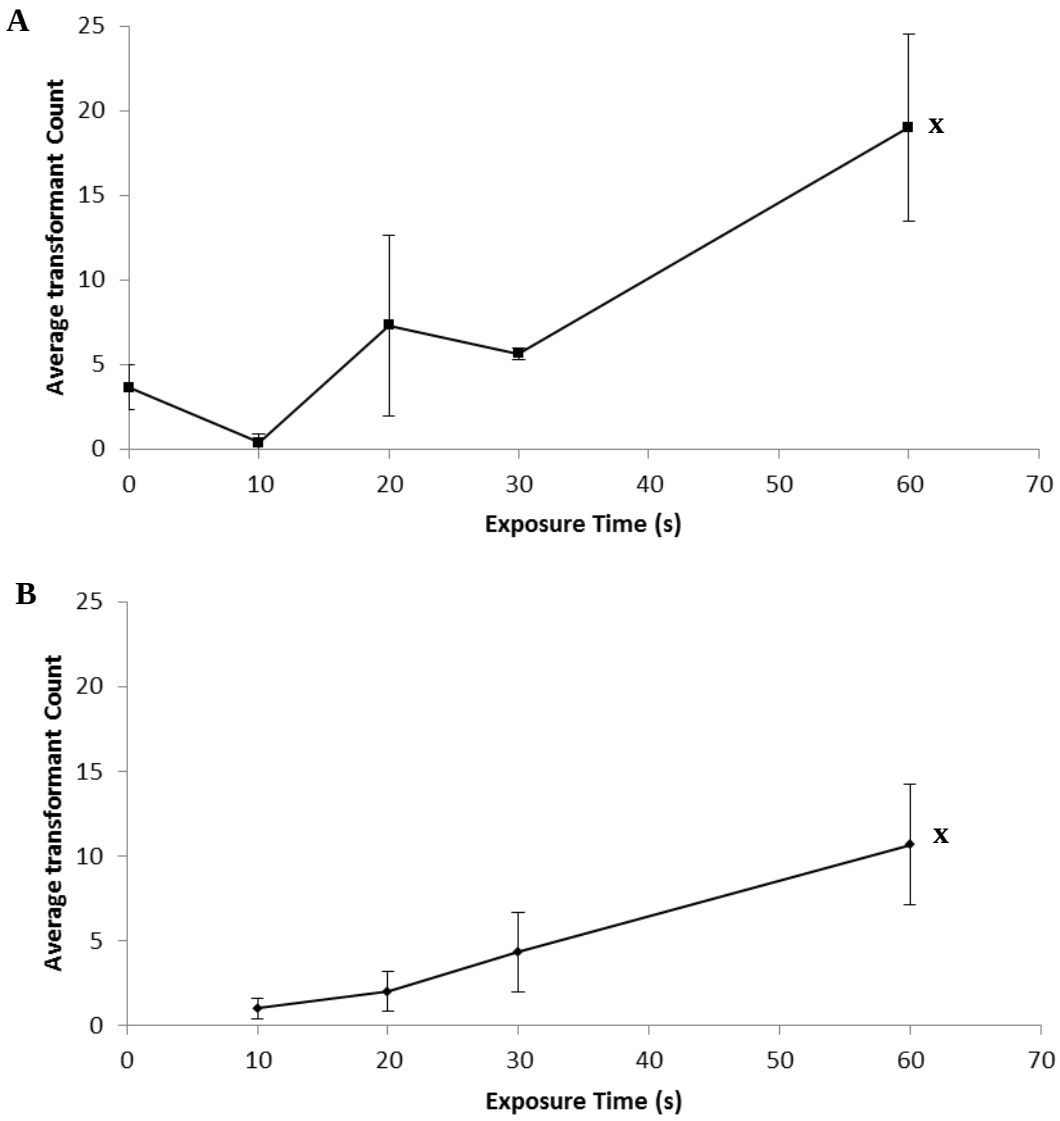

Figure 21: Transformants for increasing exposure times (A) mixed samples (B) unmixed Initial experimentation for 27.6 kHz ultrasonic exposure of 40 ml sample of P. Putida UWC1 and 100 µl of plasmid pBBR1MCS2 in 50 mM CaCl2 with three biological replicates, error bars of 1 S.E

Analysis of the data using a two-way ANOVA to compare each time point shows that for increase in the exposure time for the samples gives a significant increase in transfer rates (P=0.001) for 60 s (marked 'x' in Figure 21) against the unexposed control (0 s exposure time point). The difference between mixed and unmixed samples for the full data set, directly comparing each exposure group, gives no statistical significance (P=0.095). The variance for the mixed samples is reduced showing that mixing provides more stable and reproducible data.

Discussion

Plasmid Volume: The relationship between plasmid volume and transfer rate is expected to reach an optimum level with higher volumes of plasmids than tested, however this is experimentally restricted by the plasmid extraction resources available, making such large volumes unfeasible for this research.

The rate of transformation compared between the bath and direct immersion procedures, once adjusted for the plasmid concentration being 10 times lower in the direct immersion method, is approximately 10 fold lower, likely due to the relative reduced cell concentration in the direct immersion sample, resulting in fewer cell/plasmid/cavitation interactions. The efficiency of transfer remains satisfactory with flexibility to increase plasmid concentrations to see a resultant efficiency boost if necessary, whilst maintaining the experimental simplicity.

Mixing and Initial Exposure Time: For increasing exposure time the preliminary dataset is small, however increasing exposure gives increasing transformant counts, a peak in transformation rate has not been reached, so longer exposure times need to be investigated to identify the peak time. When compared with data from the ultrasonic water-bath it is clear that longer exposures are required for the probe due to the changes in the experimental conditions including the volume and concentration of the cell/plasmid mixture and the power and resultant change in cavitation rates and hence cavitation/cell/plasmid interactions when using the sonotrode and direct immersion procedure.

Differences between the mixed and unmixed samples are not statistically significant; however for longer time periods the graphs show a slight separation that with further investigation and increased replicates a trend may develop. Continuous mixing also increases the complexity of experimentation; due to this mixing the samples will become increasingly impractical as the experiment develops, especially as the final premise is *in situ* environmental samples where such mixing would be unsuitable.

It should be noted that the mixing also has reduced the variation in results across the replicates suggesting uneven exposure of the unmixed samples which affects the efficiency. The cause is most likely due to the shape of the containing tube with a tapered base causing varying circulation dependant on the exact alignment of the sonotrode and mixing would negate these variations.

Some mixing naturally occurs as the micro-bubbles produced are pushed away from the tip of the sonotrode by the ultrasonic sound waves then either collapse creating random micro-flows or begin to disperse to the surface allaying fears of repeated damage to cells around the tip. This mixing was demonstrating using potassium permanganate as a dye (see Figure 22), which shown complete dispersal of the dye within 2 seconds, whilst unsonicated there was no dispersal, showing that the sonication itself provides some mixing however the micro-flows may encourage the denser cells to concentrate at the base of the sample tube.

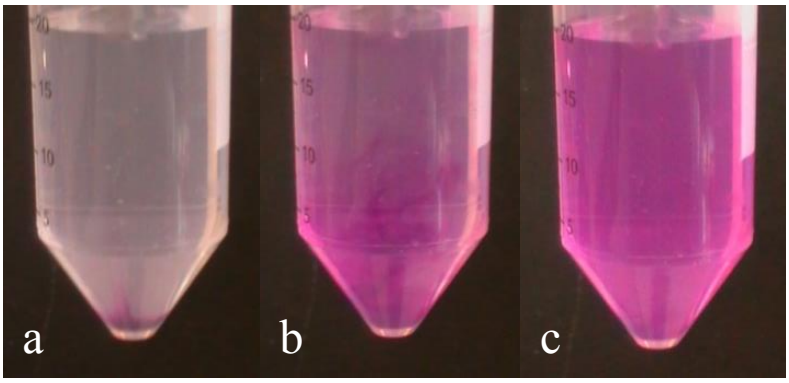

Figure 22: Sample mixing demonstrated by Potassium Permanganate ($KMnO_4$) dispersal, a) 0 s, b) 1 s and c) 2 s

Conclusions and Progression

Increasing plasmid volume directly results in increased transformation, as expected, over the range tested. The rate of increase showed that thus far plasmid concentration was a limiting factor to UGT and further increases in rate are expected with increases in plasmid concentration until plasmid concentration is no longer limiting; this limit is, however, difficult to predict due to the range of factors involved.

The practicalities of producing large volumes of plasmids for each experiment will be the limiting factor for future experimental use. Thus 50 – 100 µl is recommended dependant on the number of samples or the specificity of the experiment.

Longer exposures produce more transformants within the time ranges used in this experiment however a peak value has not been reached and increased exposure times are required to fully investigate this, potentially up to 0.5 h exposure, however by this point high cell lysis is expected from over-exposure. This will be fully investigated in future experiments.

Mixing during exposure gives more stable and reproducible results however the increased experimental difficulty outweighs this given the aim of *in situ* application and therefore should be omitted for future experiments considering the dye test showed mixing also occurred, driven by the ultrasonic waves, without exterior influence.

As time periods increase investigation into secondary effects of the ultrasound should also be considered including beneficial mixing of the cell suspension but also potentially harmful increases in temperature both of the probe tip and in the cell suspension produced as a secondary effect from the vibration of the apparatus and the energy generated in the microbubble collapse.

3.2.4: Effect of Exposure Time on Transformation Efficiency and Adjustments to $CaCl_2$ Dosing to Reduce Dilution

Abstract

The number of transformants increased as the length of sonication increased up to approximately 6 min, following this the rate of cell lysis increased to negate any further increase up to a survival of just 1% of cells after 20 min; however the relative efficiency still increased as the transformants formed a larger proportion of the remaining cells which may have uses for specific applications (i.e. *in vitro*). When focussed upon the peak transfer rate the optimum exposure time was confirmed to lie between six and eight minutes however due to technical difficulties with the apparatus nearing the end of its serviceable lifespan the data produced is sub-standard for definitive use. It did however highlight the need to control the heating of the probe and sample over the longer exposure periods. A new sonotrode must now be designed, manufactured and tested; however there is an opportunity to optimise the sonotrode design to this work and to increase its resilience to degradation of the tip. An attempt to extend the life of the old sonotrode during manufacture of a replacement a reduction in sample volume by increasing the initial $CaCl_2$ concentration and hence reduced dilution whilst maintaining the final $CaCl_2$ concentration was shown as suitable for UGT and able to reduce the exposure to 3 minutes. As the cell/plasmid/cavitation interaction has been understood to be the driver behind UGT, an alternative to direct immersion is proposed for future development: a microfluidic device, enabling control of the time period the

cell/plasmids are exposed to ultrasound by passing a controlled flow of cells and plasmids through the cavitation zone at the sonotrode tip.

Introduction

Preliminary experiments showed that increased exposure time resulted in increased transformations; however with maximum exposure of 1 min a peak rate was not reached. On reflection, with a hundredfold increase in sample volume and hence cell dilution, the exposure time could be expected to be within the range of one hundred times the length (i.e. ~16 min) as transformation is driven by the interactions between micro-bubble cavitations, cells and plasmids. However with changes to the apparatus and direct immersion of the probe reducing energy loses this time period may be reduced.

As the exposure times increase adverse effects of sonication will become more apparent namely over exposure leading to cell lysis so survival rates will need to be monitored and the highest efficiency of transfer may not be the most suitable for environmental transfer as killing most of community is counter to the aims of the project and negates one of the key advantages over other transfer methods. Secondly cavitation creates extremely localised high temperatures, whilst these quickly dissipate the residual effect over a long period will result in overall heating of the sample which may affect the transfer and as such this variable should be minimised.

Once a rough value for exposure was found, the experiment was repeated to focus on this region between 4 and 8 minutes. However the sonotrode was found to be reaching the end of its usable life and overheating during the exposures, so shorter exposure times were needed to continue use. In an attempt to reduce the length of sonication a reduced volume was suggested, rather than reducing the total sample volume in proportion, a higher concentration $CaCl_2$ was used to add to the sample, leaving the same final concentration. It is hypothesised that by dosing the cells with a more concentrated $CaCl_2$ solution but at a lower volume the optimum exposure time will be reduced. Furthermore there will be a transfer efficiency improvement as the cell/plasmid suspension will be less diluted.

Materials and Method

The direct immersion method is used as outlined previously, with 27.5 kHz ultrasound, 100 µl of plasmid pBBR1, 20 ml of overnight growth of *P. putida* UWC1, 20 ml $CaCl_2$ and exposure periods of: 10, 20, 30, 60 s and 2, 4, 8, 12, 16 and 20 min, with 5 replicates of each. Following 2 h incubation the samples were concentrated by centrifugation, however for some samples this was found to be unnecessary as the colony counts were too many to be distinguishable and they required re-diluting and re-spreading on selective media. To target the peak exposure time; periods of 3, 4, 5, 6, 7 and 8 minutes, 10 replicates were performed for each (except the 8 minute samples for which only 5 were performed due to severe overheating of the sonotrode).

In order to scale down the volume without a loss in cells 1 ml of 1 M $CaCl_2$ was added to the 20 ml cell suspension instead of 20 ml of 100 mM, resulting in the same 50 mM concentration as well as a subsequent doubling of both the relative cell and plasmid concentration. A 30 second exposure at 27.8 kHz was used with replicates in triplicate of the two $CaCl_2$ treatments.

Results

Preliminary Exposure Time

The transformant count peaks between four and eight minutes of ultrasound exposure before tailing off for longer exposures. Up to 60 seconds exposure cell death is low but steadily increases after this point up to 20 minutes where less than one per cent of the cells survive when plated on non-selective agar, around 4-8 minutes the cell lysis is noticeable without critical impact on the sample (~80% survival).

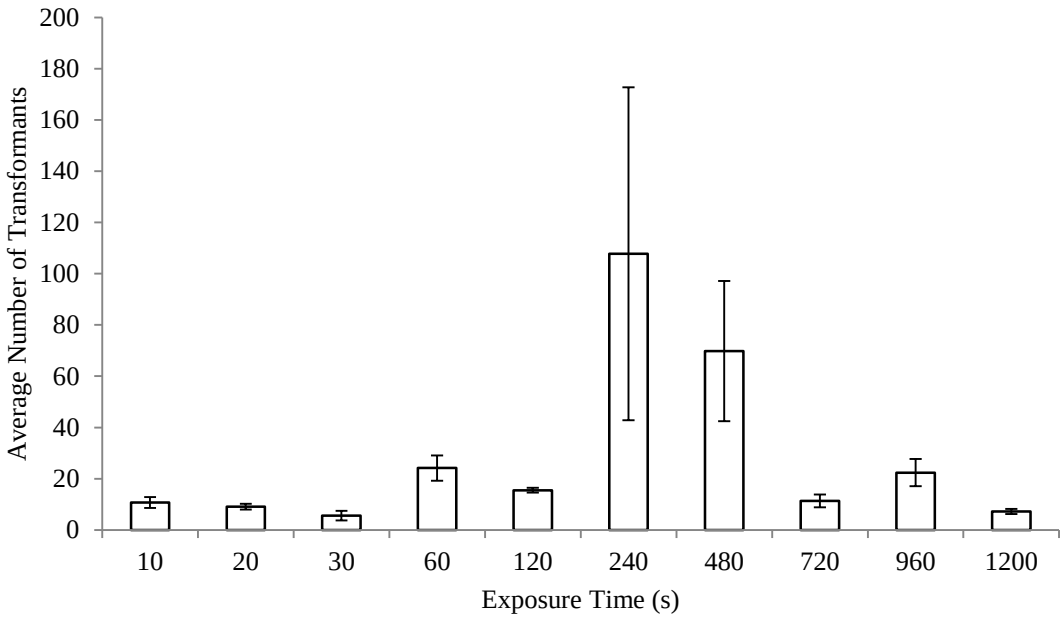

Figure 23: Average Transformant Count as Ultrasonic Exposure Time Increases mean values with 1 S.E. error bars

For over 4 minutes exposure efficiency of transformants/survival was over 10^{-7} and peaking at 10^{-6} at 16 minutes of exposure however survival rates were much lower (see Figure 24), with less than 10% of cells surviving the sonication process for 16 minutes, so the actual transformant count was decreased.

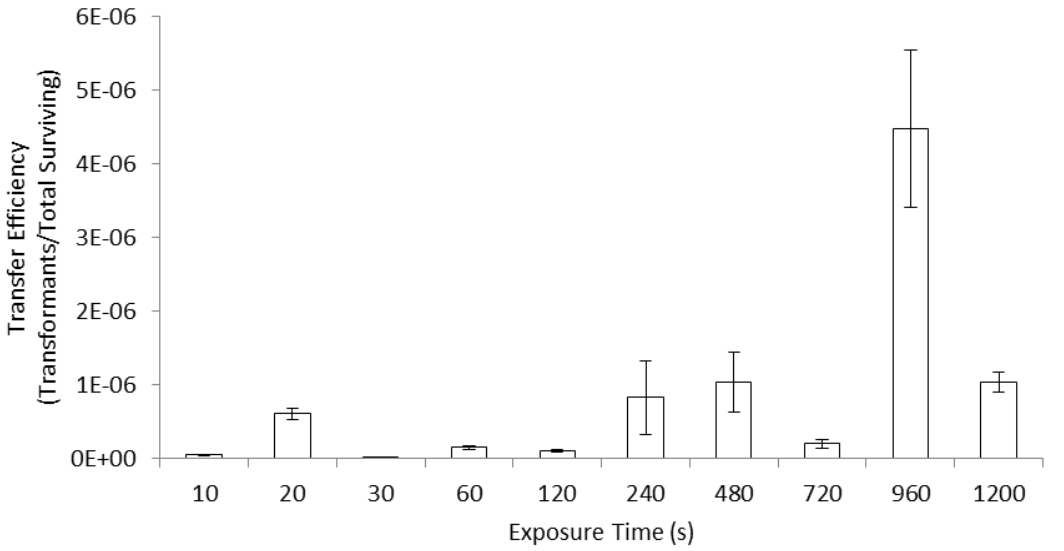

Figure 24: Transfer Efficiency as Ultrasonic Exposure Time Increases mean values with 1 S.E. error bars

During the exposure some heating was noticeable to touch, notably for exposures over 8 minutes the sample tube felt warm and the probe itself was also hot to touch, no numerical temperature measurements were taken. It was also noticeable that for the longer exposure times (>8 minutes) the cells aggregated into a gelatinous globule toward the bottom of the sample tube as if they were being forced down and together by the force of the ultrasonic waves, they remained in this way until the samples were shaken.

Targeted Exposure Time

During the exposure for each time period it was noticed that the probe and samples warmed, sometimes dramatically, during exposure with the probe becoming too hot to touch after the repetitions. The probe was allowed to cool between the time periods and ice was used to try and reduce the warming of the samples but this was not found to be particularly effective, especially over the longest exposure times leading to ceasing the testing prematurely before damaging the sonotrode apparatus.

From the data collected the transfer rates found were quite varied with the later samples for each time period producing noticeably lower transfer rates, which is attributed to the overheating of the sonotrode for later replicates in each time set. However there was an overall average increase with 6-8 minutes exposure producing a maximum number of transformants. Due to the unquantifiable effects off the overheating these results give only an indication of the optimum transfer time and will be repeated with the second generation apparatus.

Reduced Transformation Volume

The mean values for the reduced $CaCl_2$ volume are over double for those with the regular amount (see Figure 25), although the variance in the transformants for the replicates is high and analysis by ANOVA shows no significant difference ($p>0.1$). There were no differences in survival rates identified.

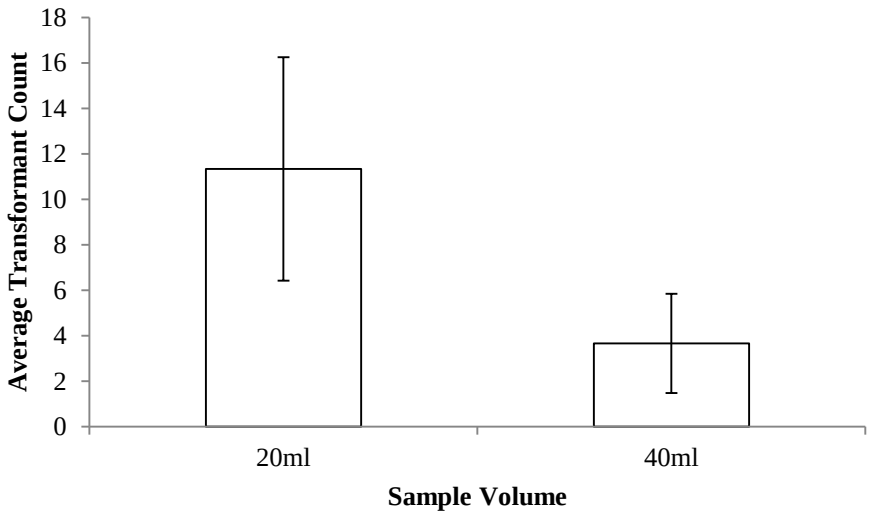

Figure 25: Effect of Reduced Volume of $CaCl_2$ Mean values with 1 S.E. error bars

Discussion

The optimum range of exposure time is much higher (~40 times) than that found with the glass vials in the old ultrasonic bath, this is most likely due to the lower cell/plasmid concentration and the differing power output of the probe to the bath, despite the reduced energy loses from direct immersion. The focal region of the probe will also be different as the bath was able to expose the majority of the sample in the vial simultaneously, the probe focuses the ultrasound and cavitation is generated over a small volume near the tip out of the whole 40 ml suspension and the time spent by the individual cells in the focal region is much reduced. The exact volume of the focal point of maximum cavitation within the sample is unknown and difficult to visually quantify due to the high speed and microscopic scale of the interactions however does lead to suggestion that the creation of a microfluidic device that allows small volumes of sample to mix with high plasmid concentrations flowing in a controlled manner past the cavitation point could provide an alternative laboratory based development path of this technology.

With increased exposure time the efficiency and rates of transfer match and even exceed those achieved in the old water bath system showing the number of cell/plasmid interactions mediated by the micro-bubble collapse has reached a similar rate despite the hundredfold cell concentration decrease and the tenfold plasmid concentration decrease.

As expected, with increased exposure time cell lysis also occurred with just 10 percent of cells surviving for the 16 minute exposure, hence for environmental samples where the aim is to minimise impact on the community this is unsuitable and the balance between transformants and cell viability is again key to the continued development of the procedure.

The mechanism of formation of the globule found for longer time periods is unknown however the separation had a clear boundary with a volume of approximately 5 ml, larger and less dense than the pellet that would form by centrifugation, but showing a density differential as it remained near the lowest point of the tube following gentle inversion, suggesting an agglomeration of organic matter that has been forced to the bottom of the tube by the repeated small impulses generated by the ultrasound waves.

When assessing the targeted results the overheating of the sonotrode proved a major hindrance and as such the data was not of sufficient quality to take specific conclusions on the precise optimum exposure time for transfer, solely that a reasonable rate of transfer was found over all the tested time periods. The final replicates at 8 min exposure were not performed due to the issues surrounding the overheating, both the temperature of the probe and sample, as well as the time delay between samples attempting to cool the apparatus leading to a difference of over two hours between the first and last samples leading to differences in cell conditions as they would continue to grow during this time making the samples incomparable.

It was concluded that the sonotrode was reaching the end of its operational lifespan as the tip of the horn had become pitted, this occurs due to normal wear and tear on the apparatus as cavitation occurs near or at the tip and damaging it. This decreases the efficiency of the sonotrode, producing more vibrations and heat, and the cavitation becomes more erratic. Hence despite minimal useful data being produced this experiment allowed greater understanding of the apparatus and its limits, and how these affect the experimental samples.

Having reduced the total sample volume and resorting to shorter exposure times to extend the life of the sonotrode; no significant change in transfer was seen between the $CaCl_2$ treatments thus the use of 1 M $CaCl_2$ as opposed to 100 mM to give a final 50 mM concentration is an acceptable change both reducing preparation of the

samples and having no detrimental effects. This is slightly surprising that the increase was not significant as with an increase in concentration of cells one would expect an increase in cell/plasmid/cavitation events and hence transfers, however the effect of the ageing sonotrode may have an influence on this.

Conclusions and Progression

The optimum exposure time lies around the 6 minute time period for a 40 ml sample, further assessment is required to confirm and narrow down the range. For differing volumes and cell/plasmid concentrations variations are expected and for longer exposures the formation of the cell globule should be monitored.

The longer exposures have provided a clear step forward to the goal of *in situ* use producing a much higher transfer rate than the initial 10 second testing which is comparable or higher than that found in the previous water bath tests and giving confidence that developments will be able to improve this efficiency.

Further investigation needs to be undertaken into the effects of heating during exposure of the samples and to find a suitable method to reduce the heating or improve the cooling of the sample and probe if there are significant negative effects. However for a single environmental sample this will be much less of an issue as the heating over a single exposure is much less than after repeated runs.

A replacement sonotrode needs to be designed, manufactured and tested for future experiments. The design will be optimised for use around the cavitation frequencies, specifically 27.5 kHz, and produced with replaceable tips and from titanium as opposed to aluminium to maximise the operating lifespan.

Testing using a lower $CaCl_2$ dose at a higher concentration was effective in enabling transfer at a reduced total volume and also reduced exposure time to enable continued use of the sonotrode whilst waiting for the sourcing of the parts and manufacture of the apparatus. A sonication concentration of 50 mM $CaCl_2$ is still recommended, using a 1 M initial concentration to reduce the final sample volume allowing for shorter ultrasound exposures.

Investigation into the concentrations and limiting factors of UGT should be further investigated to understand and improve the mechanisms of UGT. Construction of a microfluidic device to sonicate a small flow of cell/plasmid suspension as opposed to

sonicating large volumes has been highlighted as an alternative pathway for development whilst still being applicable to environmental samples. This thesis, however, continues to focus on the direct immersion method and the design, construction, testing and application of a microfluidic device could provide a standalone project of its own.

3.3: Second Generation Sonotrode Testing

The design sourcing and manufacture of the second generation sonotrode was a lengthy process (see Section 2.3) however once received it was necessary to commission it and test its operational efficiency, unfortunately by this point the previous model was barely functional and direct comparison was not possible. However the wealth of data previously collected allowed for adequate evaluation of the performance prior to further experimental development.

3.3.1: Optimum Frequency Analysis/Confirmation of Cavitation as the Driver for UGT

Abstract

The second generation sonotrode effectively generates cavitation bubbles at the three tested cavitation frequencies and these successfully mediate UGT at a comparable level to the previous model. The cavitation frequency found around 30 kHz is shown to be most effective at transfer for these experimental conditions and cell lysis is not excessive. Re-optimization of the exposure time for the new apparatus and adjustments to the experimental procedure are however still required.

Introduction

Once manufactured the first stage of commissioning the new sonotrode was to test the optimum transfer frequency as the changes in horn material and geometry could affect how aggressively the cavitation is produced. Combined with this there is an opportunity to assess the sensitivity of the sonotrode to frequency for cavitation generation by testing a few kHz either side of the cavitation frequencies.

The method was also adjusted to increase the cell concentration: 1 ml of 1 M $CaCl_2$ was used instead of 20 ml of 100mM maintaining ~50 mM final concentration but nearly doubling the cell concentration as shown in the final experiments with the first generation sonotrode, this should both increase transfer efficiency whilst

reducing waste and manipulation. The exposure time is correspondingly lower at 3 minutes to prevent cells becoming over-exposed and being lysed, the time will be re-optimised in a later experiment.

Materials and Method

Ultrasonic transfer experiments used the plasmid pBBR1MCS2 as the transferred genetic information, containing a Kanamycin resistant gene to act as a marker and *Pseudomonas putida* UWC1 as the recipient strain. The plasmid was grown in *Escherichia coli* K12 DH5α and prepared using a Qiagen Maxiprep kit (QIAGEN, UK) as per the manufacturer's protocol. 20 ml per sample of *P. putida* was grown overnight at 27 °C in LB broth, 1 ml of 1 M $CaCl_2$ was added and 20 µl of plasmid (~8 ng/µl) was added. This was exposed for 3 min to 3 cavitation frequencies of: 24, 30 and 41 kHz ultrasound (located prior to exposure on the day to ensure these were the peak cavitation frequencies) and plus and minus 1 kHz for each cavitation frequency; for each period 5 replicates were performed. All work was performed at room temperature (21 °C) unless otherwise stated. Samples were then incubated for 2 h at 27 °C to allow for recovery then 100 µl of sample was spread on LB agar plates with 50 µg/ml Kanamycin supplement for transformant selection. Samples were also diluted $x10^{-6}$ and spread on LB agar plates with no selective pressure to assess cell survival. Resultant colonies were counted after overnight growth.

Results

Transformation is, as expected, only seen at the cavitation frequencies with 30 kHz being the optimum for both transformant counts and transfer efficiency. Cell lysis by the ultrasound is notably higher at 30 and 41 kHz whilst at the other frequencies including at the 24 kHz cavitation frequency the lysis is less prevalent.

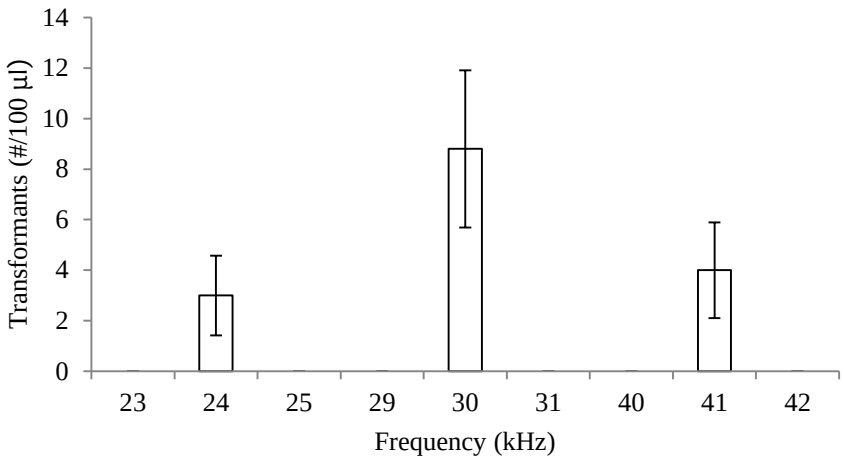

Figure 26: Transformant Count for Cavitation and Non-cavitation Frequencies (2^{nd} Generation Sonotrode) mean of 5 replicates, error bars of 1 S.E.

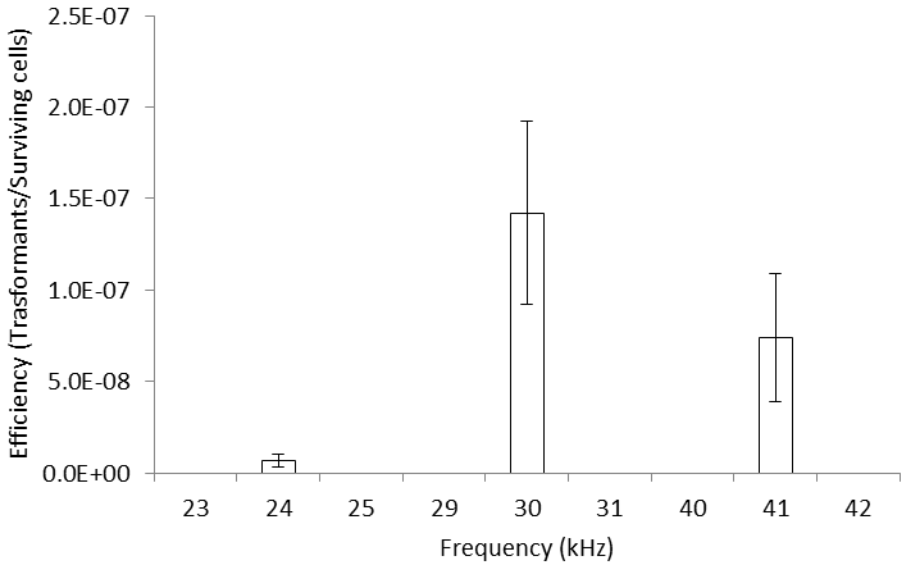

Figure 27: Transfer Efficiency for Cavitation and Non-cavitation Frequencies (2nd Generation Sonotrode) Ratio of average transformant count over total survival count, mean of 5 replicates, error bars of 1 S.E.

It was also noted that during sonication it was harder to maintain the cavitation frequency at 24 kHz as the function generator was unable to hold the frequency to sufficient accuracy of less than ± 0.1 kHz at this level and the cavitation frequency was very precise. The other cavitation frequency peaks were less precise (± 0.2 kHz) and where able to be generated and maintained with more stability.

The data was analysed by ANOVA, showing statistical difference, $p<0.001$, between the groups with cavitation and those without. Analysing the individual frequencies by ANOVA and using a post-hoc Tukey test it was shown that the transformant count at 30 kHz was statistically distinct from the other groups, $p=0.001$.

Discussion

The second generation sonotrode is shown to be successful at mediating UGT under similar parameters to the previous one, with reasonable efficiency, and cavitation is seen to be the driver also providing confirmation of the hypothesis through this reproducibility across equipment.

Both statistical and graphical analyses clearly show perfect correlation between cavitation frequencies and gene transfer. Whilst the precise mechanism is still unknown this provides conclusive evidence that the cavitation effect has a key role in UGT, lending credence to the hypothesis that the collapse of the micro-bubbles formed by cavitation creates temporary pores in the bacterial cell walls allowing DNA to enter. To be able to image this process would be ideal, however a camera which had sufficient resolution and frames per second could not be sourced to account for the scale and vibrations generated by the ultrasound, whether it would be possible to fix the cells immediately after sonication to prevent any morphological changes and then examine them with electron microscopy was not investigated but suggested for future study.

Conclusions and Progression

The second generation horn is effective at transferring plasmid DNA into cells by ultrasound and therefore suitable for continued use.

UGT is conclusively shown to be mediated by cavitation; with the optimum frequency under these conditions of 30 kHz. The cavitation frequency close to this value is recommended for all further work using this procedure, and the exact frequency for the given conditions should be located immediately prior to sonication using a control sample.

To further understand the mechanism used imaging of the process would be highly beneficial either by adapting the apparatus to use with a high speed microscope or render the cells inert following sonication for study under an electron microscope.

3.3.2: Optimum Exposure Time for Reduced Volume Sample

Abstract

A reduction in volume of sonicated cell suspension brings a corresponding reduction in optimum exposure time (3 minutes) however the resultant increased cell/plasmid does not produce a statistically significant increase in transformation. The shorter exposure time and upgraded sonotrode no longer result in noticeable heating of the sample but monitoring is still necessary.

Introduction

Having adjusted the procedure to reduce the experimental sample volume, by adding $CaCl_2$ at a higher concentration whilst maintaining the same final concentration, it is hypothesised that the optimum exposure time will also be reduced. As the volume is lower the sample will be exposed to the sonotrode focal point more often over the exposure time increasing the likelihood of overexposure and hence cell lysis, to mitigate this shorted time periods will be tested. In the previous experiment to confirm the optimum frequency 3 minute exposures were used as an estimated reduction, having previously assumed an approximate linear relationship between exposure time and cell concentration. 3 minutes gave acceptable results however confirmation over a range of time points is required.

It is hypothesised that for the reduced volume procedure approximately 3 minutes will give the optimum transfer rate without high rates of cell lysis.

Materials and Method

The method followed the reduced volume, 20 ml, direct immersion method with ultrasound exposure frequency of 30 kHz and 20 µl of plasmid pBBR1 added. Samples were exposed for 2, 3, 4, 5 and 6 minutes, with 5 replicates for each, negative controls with no plasmid were exposed for 3 minutes and no ultrasound controls were un-exposed.

Results

Optimum exposure time peaks around the estimated 3 minute mark, however it is not significantly higher than the 2 minute exposure. For the 3 minute exposure around three quarters of cells remained viable (570 CFU’s against 800 for the no sonication

control). By 4 minutes over half the cells were no longer viable and by 6 minutes less than 3% of cells were viable.

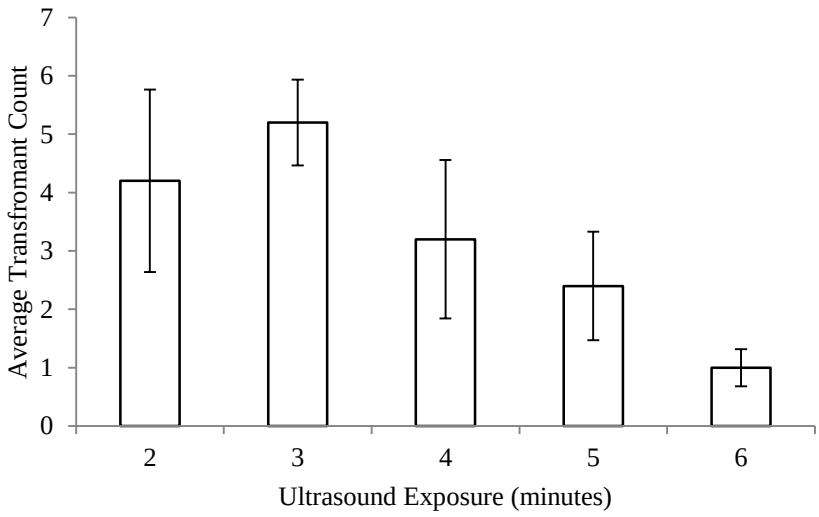

Figure 28: Optimum Exposure Time for UGT Mean of 5 replicates, error bars 1 S.E

Warming of the probe was noted for the longer exposures however for the shorter exposures only the transducer became hot and the heat was not conducted to the tip or into the sample.

As the focus of the work is on *in situ* application the transfer rate the efficiency is not shown for each exposure due to the longer efficiencies resulting in higher cell lysis which would excessively damage the community, therefore the rate of transfer is a more reflective data set given the starting cell samples are drawn from the same growth batch, however for the 3 minute exposure efficiency is of the order 10^{-7}.

Discussion

2-3 minutes exposure gives not only a relatively high transfer rate but also low cell lysis making it very suitable for future investigation into UGT using the sonotrode, the sonotrode also remained cool at the tip however the transducer still warmed noticeably and this should be carefully monitored as if it does not cool between exposures the heat may conduct to the tip despite the low conductivity of the titanium tip section.

The transfer efficiency for the optimum exposure remains around 10^{-7}, comparable to previous experiments.

It is also clear that with a reduced volume and the new sonotrode that over exposure to sonication lyses the cells which may become important when attempting transfer to other species which have differing cell wall structures and strengths. Especially as shorter exposures have still mediated transfer, albeit at a lower level, and once an advantage occurs selective pressure will enable the plasmid to reproduce and become dominant in the species.

Conclusions and Progression

The optimum exposure time for the reduced volume sample and ~30 kHz is 3 minutes; this is variable on the experimental conditions but provides a strong base for the final protocol. Over exposures are rapidly damaging to the viable cell counts and should be avoided unless required for specific work.

This series of experiments forms the base mechanical parameters for the sonotrode procedure, covering iterative developments of: frequency, time, chemical concentrations and volume to generate an effective and robust procedure to be applied to optimising the biological parameters and progression toward environmental samples.

3.3.3: The Effect of OD, Growth Phase and $CaCl_2$ Mixing on the Rate of UGT

Abstract

Across the range tested OD has no significant effect of UGT rates, combining this with reaching an optimum exposure time and hence amount of cavitation, the remaining limiting factor from the cell/plasmid/cavitation triumvirate must be the plasmid concentration. Thus where possible the highest plasmid volume practical should be used. For the preferred growth phase; the stationary phase bacteria gives the maximum transfer rate. This ideally places this technology as suitable for environmental samples in naturally stable ecosystems, as it does not require the cells to be in the exponential growth phase. Throughout the work $CaCl_2$ has been used to improve transfer, the ‘Calcium Effect’ is known to improve many lab based transformation methods and this is also true for UGT. Here the effect of pre-mixing

the calcium ions with the plasmid or the cells to maximise dispersion and interaction has been shown to have a negligible effect. However ionic concentrations will need to be accounted for when performing UGT on environmental samples.

Introduction

Previously using an increased concentration of $CaCl_2$ to reach the 50 mM concentration and hence reducing the dilution of the final cell plasmid suspension did not produce a statistically significant difference in transfer. This seemed counter-intuitive unless there was another limiting factor to UGT so a deeper investigation was undertaken to assess any variations that might be caused by lower cell density (OD600) or whether the growth phase of the bacteria had any impact of transfer efficiency. OD600 was selected as the measure for cell density as it provides a quick and reliable result whereas other methods such as microscope field counts would result in increased lag between counts and sonication given the number of samples being tested.

It has been suggested that as the hypothesised mechanism of transfer is from reversible poration of the cell walls (where the ultrasound damages in the cell walls to allow transfer but are able to recover (Deng et al., 2004)), that the growth phase of the bacteria might influence UGT. The key areas of difference are expected to be between the exponential phase and the stationary phase, with either the cells in the exponential phase being more susceptible to poration but correspondingly more able to recover or those in the stationary more stable and less likely to be irreversibly damaged. By generating a growth curve and performing transfers at time gaps along the curve an understanding of any differences can be identified.

One facet of current gene transfer methods is the use of calcium chloride as a mediator to improve transfer rates; however the process behind this ‘Calcium Effect’ is not well understood. It is assumed the polar molecules negate the charge of the DNA and cell wall allowing for closer interaction and hence increased chance of transformation. It is hypothesised that there could be a differential between whether the $CaCl_2$ is pre-mixed with the plasmid prior to addition to the cell suspension, allowing binding by van der Waals forces, or whether mixing the $CaCl_2$ with the cells prior to adding the plasmids, where it can attach to the cell wall, provides a

better environment for UGT (Zhou et al., 2008, Yoshida and Sato, 2009, Dagert and Ehrlich, 1979).

Materials and Method

The reduced volume direct immersion method was used; 20 ml of *P. putida* UWC1, 1 ml of 1 M $CaCl_2$ and 20 µl of plasmid were sonicated for 3 minutes at 28 kHz. Overnight growth of *P. putida* UWC1 was, respectively, diluted and concentrated to concentrations of 0.25, 0.5, 1, 2 and 4 times, OD600 was measured using a Biochrom WPA Biowave S2100 Spectrophotometer (Biochrom, UK).

To assess the growth phase a bacterial growth curve of OD600 was produced; *P. putida* UWC1 was grown overnight in LB broth, a 1 ml inoculum was added to 20 ml of fresh LB broth, mixed and decanted into 10 wells of a clear 96 well micro-plate. OD600 was then read over 18 h using a Synergy 2 Micro-plate reader (BioTek, US), at 30°C with intermittent shaking.

From the curve generated time intervals to include each growth phase were selected as: 0, 1, 6, 8 and 12 hours. Sonication was performed at 28.7 kHz, for 3 min, on 20 ml of cell growth with 1 ml of 1M $CaCl_2$ and 20 µl plasmid pBBR1MCS-2, with 5 replicates; OD600 was measured prior to sonication.

For investigation of $CaCl_2$ binding; direct immersion procedure was followed, however following decanting overnight growth into 20 ml aliquots, half the samples had 1 ml of 1 M $CaCl_2$ added and mixed followed by the plasmid, the others had a pre-mixed mixture of 1 M $CaCl_2$ and plasmid added, both producing a final concentration of 50 mM as previous shown to be most efficient (Song et al., 2007). 3 replicates of each were performed as were negative controls with no plasmid and no ultrasound.

Results

Effect of OD: Whilst the dilutions linearly affected the OD600 as expected, there was no significant variation in transformation between dilutions tested.

Table 3: Transformant Rate for Differing OD Dilutions

Concentration/ Dilution Factor	OD600	Average Transformant Count
0.25	0.08	0.667
0.5	0.197	1
1	0.383	1.666
2	0.908	1
4	1.393	1

Bacterial growth curve and growth phase: From the OD 600 data the lag phase is estimated to approximately 4 h, followed by the exponential phase until circa 10 h and stationary from the onwards, no death phase was reached within the timeframe of this experiment (see Figure 29). Time points for UGT were selected to include each phase (0 and 1 h – lag phase, 6 and 8 – exponential and 12.5 h stationary).

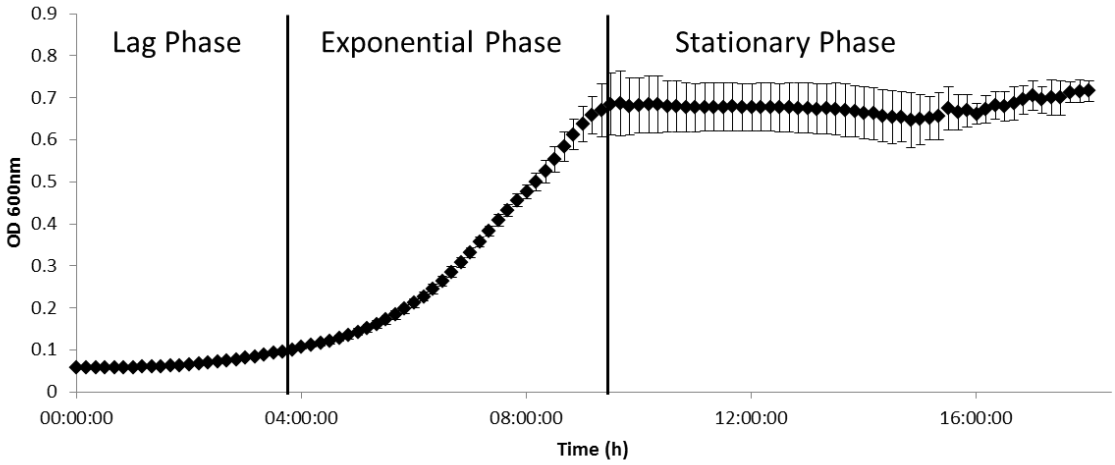

Figure 29: Growth Curve for *P. putida* UWC1 Mean of 10 replicates at OD 600, 1 S.D. error bars

Over time the transfer rate increases with 12.5 hours (the stationary phase) being significantly higher than 0.25 and 1.25 hours (lag phase), (by ANOVA $p<0.05$) however the difference between exponential growth and stationary is not shown to be statistically significant.

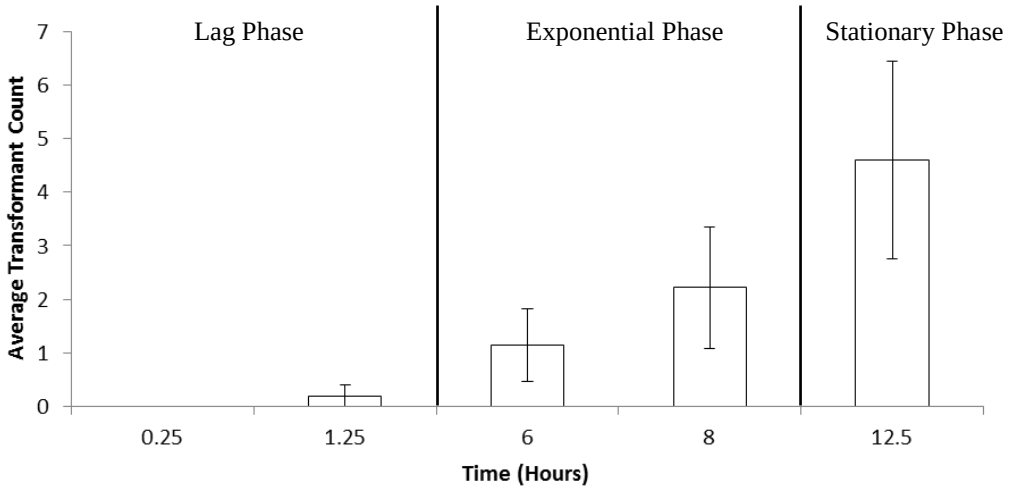

Figure 30: Transformation Rate for Differing Growth Phases Mean of 5 replicates, error bars of 1 S.D.

$CaCl_2$ Binding: The differential between the results is minimal and within one standard deviation. Furthermore, following analysis by ANOVA, the difference is not shown to be statistically significant, $p>0.05$.

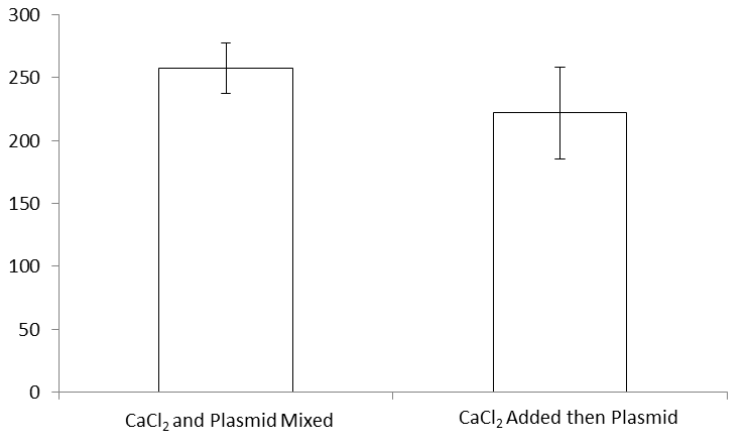

Figure 31: Effect of Calcium Mixing Mean of 3 replicates, error bars of 1 S.D.

Discussion

Cell Concentration: The lack of variation despite a 16-fold difference in cell concentration strongly points towards another limiting factor on the rate of transformation, however for all samples few transformants were produced reducing the data quality and significance of any conclusions.

Whilst this lack of variation is slightly surprising, when reflecting on the results in conjunction with previous experiments (showing plasmid concentration not being maximised), this also confirms the lack of statistical significance found between concentrations used in Section 3.2.4 testing the effect of increasing concentration by reducing $CaCl_2$ volume, and also that when assessing plasmid concentration no maximum was reached. Combining these findings with the previously shown peak transfer rate for exposure and hence peak cavitation events has been reached with 3 minute exposures; plasmid concentration is the remaining limiting factor.

Growth Phase: The stationary phase is shown to transform better than the lag phase for UGT however the difference between the exponential and stationary phase is not significant at a $p<0.05$ level ($p=0.061$). No correction for OD has been made following the conclusions above that the OD does not have a significant effect over the range of 0.1-1.0, whether there would be an effect at higher plasmid concentrations is unknown. This lack of differentiation between the exponential and stationary phases shows no advantage of targeting cells in the midst of division and that during the stationary phase the stable cells may be more resilient and able to absorb the plasmid following sonoporation and recover more successfully from the cavitation damage.

$CaCl_2$ Binding: Despite there being potential difference in binding between the $CaCl_2$ and the plasmids/cell wall, no significant difference in transfer was seen suggesting the 'Calcium Effect' is quickly disseminated and affects the samples no matter which order it is added prior to sonication. Further reading suggests longer incubations may improve rates of UGT rather than dosing just prior to sonication (Dagert and Ehrlich, 1979).

Conclusions and Progression

The experimental limits are being reached across the parameters, OD is shown not the limiting factor behind this UGT procedure within this range, however at

markedly lower cell concentrations it may become critical; cavitation has also been optimised, however a peak plasmid volume has not; these result in plasmid concentration being the limiting factor behind this UGT however dramatically increasing plasmid volume for high replicate tests is impractical, so the highest practical plasmid volume should be used.

Stationary phase is shown to be the most effective phase with the exponential phase also showing transformation. This conclusion allows the continued use of overnight bacterial growth of *P. putida* UWC1 to be used and adds validity to previous experimental work that the growth phase used was suitable for optimal UGT.

The order of calcium ion addition makes no significant difference to UGT rates and as such should be performed as is most convenient. However an investigation into the longer term effect of pre-dosing with $CaCl_2$ ahead of sonication warrants further study.

When progressing to environmental samples the OD may become limiting as cell density may be markedly lower and different species will be more or less susceptible to sonication damage and consequently further testing of lower cell concentrations is recommended. The stationary phase being the preferred growth phase for UGT is beneficial as environmental samples will usually be found naturally in a stable stationary phase. In respect to $CaCl_2$ volume it is recommended, when using environmental samples, to ensure suitable ionic conditions are present for optimum transfer.

4: Experimental Progression toward Environmental Samples

Having developed and optimized a standard procedure for millilitre scale volumes for UGT the progression towards creating a viable application continues; the aim being to directly introduce hydrocarbon pollutant degrading genes into environmental samples with minimal manipulation.

This section covers the trials, developments and limitations encountered while attempting UGT using low nutrient media, varying species and environmental samples. Ultimately most of the transfers to environmental samples were unsuccessful; this was suggested as being due to insufficient cell/plasmid/cavitation events due to low cell concentrations, interference from debris in environment samples, failure of the cavitation to penetrate the cell walls or natural incompatibility of the species and plasmids. It is suggested that for future tests an attempt to use *P. putida* UWC1 as an internal control dosed into the environmental sample to cross-check transformation conditions providing the UWC1 is able to survive in the environmental samples.

4.1: Transformation Using Minimal Media as the Growth Media

Abstract

P. putida UWC1 grown in minimal media was not shown to be transformed by UGT; whilst this line of research cannot be discounted from these results any transfer efficiency will be extremely low as has been seen by previous work. The low nutrient content and differing salt levels especially calcium salts are hypothesised as the main causes, there may also be phenotypic differences to the cell structure, these will affect both the transfer and recovery stages. Notwithstanding the low efficiency even a few transformants will affect a community and selective pressure will allow them to prosper and consequently the desired goal can be achieved.

Introduction

The first stage of the progression to *in situ* environmental samples is to test the procedure under low nutrient conditions that mimic those found in environmental samples. This allows investigation into the ability for species to recover from sonoporation under less favourable conditions and whether they still uptake and

express the plasmid. Existing transfer methods rely on high nutrient conditions for cell recovery, including the addition of SOC following transformation (Song et al., 2007). It has been shown that UGT does not require this SOC dosing for the direct immersion procedure (see Section 3.2) although it had been beneficial for the ultrasonic bath method but not imperative.

Previous work performed by the author, prior to the start this study, had shown low efficiency of transfer (~10^{-9}) using minimal media (Boardman, 2008), however this was performed using the ultrasonic waterbath procedure with glucose as the carbon source and with SOC to aid recovery.

Materials and Method

P. putida UWC1 is grown overnight in Minimal Media with 20 mM Sodium Succinate as the carbon source (filter sterilised), controls are grown overnight in LB broth. UGT is performed using the first generation sonotrode, the 40 ml final sample with 100 µl of plasmid pBBR1MCS-2 and 50 mM $CaCl_2$ is exposed for 6 minutes at 27.5 kHz. Following sonoporation samples incubated for 2 hours in the transfer media then were plated on LB agar with 50 mM kanamycin. Colonies were counted after overnight incubation. Negative controls with no plasmid added or no ultrasound were performed as well as positive controls using the 40 kHz waterbath. (Note: the low volume procedure had not been developed by this point hence the later optimised conditions were unknown)

Results

No transformants were seen from any of the MM samples; whilst 9 out of 10 of the LB samples produced transformants (see Figure 32). As the number of transformants was expected to be lower the MM samples were concentrated 10 times however following plating these no transformants were seen either. The same was true for the waterbath samples with all three LB samples resulting in transformants with none from the MM samples.

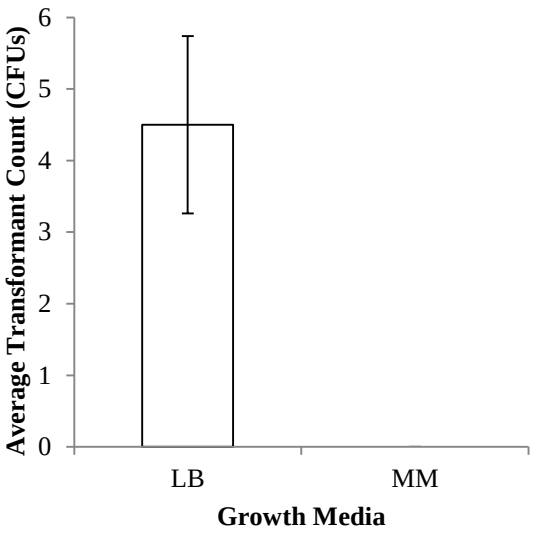

Figure 32: UGT in *P. putida* UWC1 grown in LB broth vs Minimal Media
Average of 10 replicates with 1 S.E.

The differences between the treatments are clear and statistically distinct (p=0.002, ANOVA).

Discussion

This result is a blow to the experimental development as no transformants were produced on the plates and transfer efficiency using minimal media with sodium succinate for growth must be at least a hundredfold lower than using LB (MM efficiency < 10^{-10}). Whilst transformation cannot be ruled out in MM, for any transfer the efficiency is extremely low, however a major potential benefit of UGT is that even with low efficiencies by transforming *in situ* with a selective pressure the advantage conferred to the transformed cells will enable them to prosper over untransformed competitors.

The reason behind the disparity is unclear; however the low nutrient levels are likely to be a major factor reducing the cells ability to recover from damage caused by sonoporation (Addgene, 2015); it should be noted that previous work that used SOC to aid recovery did see successful transformation at 10^{-9} efficiency (Boardman, 2008). The stress conditions may also hinder uptake of the plasmid or its subsequent expression as the cells resources are prioritised for survival. Low nutrient levels will also restrict growth prior to sonication however as previously shown cell concentration is not the limiting factor for UGT under these conditions (see Section

3.3.3) The growth media may also result in phenotypic changes affecting the cell morphology and specifically wall structure altering the impact of cavitation in mediating transfer. Previous experimentation has shown the importance of the calcium concentration on transfer rates and consequently the salt content of the minimal media relative to the LB broth prior to the dosing with 50 mM $CaCl_2$ may have affected binding and uptake of the plasmids.

Conclusions and Progression

Minimal media is not shown to produce transformants under these experimental conditions, and whilst transfer by UGT into cells grown in MM cannot be entirely discounted, the efficiency can only be extremely low. To investigate whether there is any transformation the use of a selective pressure and sampling over a time period is suggested to see whether they become dominate despite the low transfer rate. Attempting to increase the efficiency using high nutrient growth media immediately following sonication, as used in prior work where UGT was seen, would be counter-productive and a regression from the goal of *in situ* bioremediation.

Investigation into the impact of the different salt concentrations on the cells, both phenotypically and on the uptake of DNA would also benefit the understanding of the differences shown.

4.2: Transfer of Alternate Plasmids including pBAV1K into *P. putida* UWC1

Abstract

Notwithstanding a low efficiency all three further plasmids attempted were successfully transferred into *P. putida* UWC1 by UGT and the marker genes were suitably expressed. This bodes well for the introduction and expression of degradation genes into at least this species. The difference in efficiency relative to the pBBR1MCS-2 positive controls is unclear as the plasmid sizes were similar or smaller and were expressed in *P. putida* showing no plasmid compatibility issue with the species used. The low efficiency, whilst not ideal, is not a major impediment as providing a selective advantage is gained by the introduction of the plasmid this will allow the transformants to become more dominant in the ecosystem; the key advantage of the goal of *in situ* application.

Introduction

Due to the initial struggles to introduce plasmid pBBR1MCS-2 when the bacteria were grown in minimal media, it was decided to refocus the research toward transferring a range of plasmids into *P. putida* UWC1.

A series of plasmids were investigated including two gentamycin resistance plasmids, originally from a *Rhodococcus* strain (p257-1 and p259, obtained from Chen, Y., University of Warwick, UK) and a green fluorescent protein (GFP) producing plasmid, pBAV1K-T5-GFP (Km^r), (Bryksin and Matsumura, 2010). These plasmids will enable confirmation that UGT is not restricted to pBBR1MCS-2 and provide alternate selection criteria to isolate transformants, the GFP gene being especially useful for environmental samples where it can be visualised in unculturable cells under the microscope or in culturable cells under a UV light source.

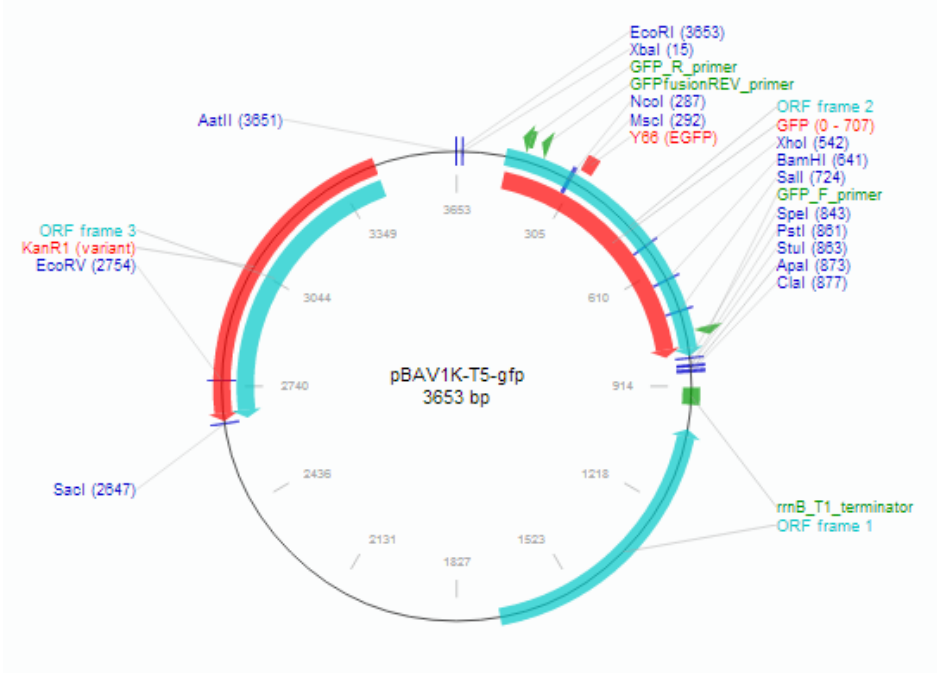

Figure 33: Plasmid Map of pBAV1K (https://www.addgene.org/26702/)

Due to the ageing of the first sonotrode and the delays in sourcing and manufacture of the second generation sonotrode these experiments were performed using the 40 kHz waterbath or using shortened exposures with the first generation sonotrode.

Materials and Method

The standard ultrasonic waterbath procedure was followed for the transfer of p257-1 and p259, using 5 ml overnight growth of *P. putida* UWC1 concentrated to 200 µl by centrifugation and $CaCl_2$ added to final concentration 50 mM, 10 µl of plasmid was added (plasmids were prepared from *E. coli* and extracted using Miniprep kit (Qiagen, UK), following the manufacturers recommended procedure). 5 replicates were performed as well as negative controls. Sonication was performed on the samples in flat-bottomed glass vials at 40 kHz in an ultrasonic waterbath.

For pBAV1K (Addgene plasmid 26702, see Figure 33) the first generation sonotrode was used with 20 ml of *P. putida* UWC1, supplemented with $CaCl_2$ to 50 mM concentration. 20 µl of plasmid pBAV1K (prepared by Qiagen Miniprep) was added and the samples sonicated by direct immersion of the probe for 30 s. Samples were performed in triplicate with negative controls and positive controls using plasmid pBBR1 as a comparator. Samples were plated on selective media and incubated overnight, the remaining sample was stored overnight in the fridge, following no growth on the test samples this was concentrated a hundredfold by centrifugation and re-plated.

Results

Plasmid transfer for both experiments was successful in the positive controls using pBBR1, confirming the conditions and apparatus were suitable.

The p257-1 and p259 plasmids produced relatively high colony counts on 5 µg/ml Gentamicin plates however the negative controls with no plasmid or ultrasound also showed similar colony counts and were not shown to be statistically significant ($p>0.05$). Selection using 10 µg/ml Gentamicin produced a single colony for each plasmid across the replicates.

Table 4: Transformant Counts for Plasmid p257-1 and p259 by UGT with pBBR1 controls

Antibiotic (conc. µg/ml)	Replicate	p257-1	p259	No Plasmid	No Ultrasound
Gentamicin (5)	1	64	56	17	45
	2	57	38	-	34
	3	60	23	-	-
	4	45	38	-	-
	5	11	82	-	-
Gentamicin (10)	1	0	1	0	0
	2	0	0	-	-
	3	0	0	-	-
	4	0	0	-	-
	5	1	0	-	-

Antibiotic	Replicate	pBBR1
Kanamycin (50)	1	1
	2	1
	3	2
	4	3
	5	9

The samples which were sonicated with pBAV1K showed no initial transformants however following concentration of the sonicated samples transformants were seen on one of the selective kanamycin plates and strongly expressed GFP both under UV light and to a level that the colouration could be seen under natural light.

Table 5: Transformant Count for Plasmid pBAV1K by UGT with pBBR1 controls

Replicate	Plasmid pBBR1	pBAV1K	pBAV1K (conc.x100)	No Plasmid	No Ultrasound (pBBR1)
1	20	0	8	0	0
2	11	0	0	-	-
3	3	0	0	-	-
LB (control)	1x10^9	1x10^9	1x10^9	1x10^9	1x10^9

Discussion

All three plasmids tested were introduced successfully however at a markedly lower efficiency than pBBR1 ($10^{-9}/10^{-10}$ efficiency versus 10^{-8} for pBBR1), this is not unexpected as pBBR1 was selected for the initial investigation as it had been designed to be a small plasmid with a wide host range hence making it very suitable for UGT. Plasmids p257-1 and p259 both originated in *Rhodococcus* species and may therefore have reduced compatibility with the *Pseudomonas* species used here. Plasmid size might be expected to also have an effect on transfer rates, similar to the linear relationship between plasmid size and transfer rate found in the heat shock transfer method (Hanahan, 1983), the control plasmid pBBR1 has a size of ~5 kb however pBAV1K is smaller at 3.6 kb and has also been designed for a broad host range, therefore the discrepancy with the control, despite identical treatment, is unexplained barring reduced compatibility however the high level of GFP expression makes this unlikely.

The colony counts seen in the 5 µg/ml of Gentamicin samples and lack of difference with the negative controls leads to the conclusion that the critical concentration for overcoming the natural resistance of *P. putida* to Gentamicin, which was uncertain prior to the work, had not been reached, however the lack of colonies on the negative controls for 10 µg/ml show that this is the effective selection concentration.

Conclusions and Progression

The three plasmids were successfully transferred into *P. putida* UWC1 by UGT however at a reduced efficiency compared with pBBR1. Whether adjustments to the procedure could increase the rate is unclear, however even a reduced rate is beneficial providing a selective advantage is gained by the transformants over the remainder of the community. To demonstrate the effectiveness of this advantage investigation into the transformed cells becoming dominate is recommended as a priority over attempting to increase transfer rate for each individual set of plasmids used.

4.3: Domination of Sample Species by Transformed Cells following UGT

Abstract

UGT successfully mediates transfer of plasmid pBAV1K in the bacteria and despite a low efficiency incubation of the samples with selective pressure at a lethal level and no other manipulation allows the transformed cells to replicated and become the dominate species within 7 days. For lower selective pressures the transformants are maintained and able to replicate reaching a stable proportion of the total sample within 2 days. This provides an excellent base for use in environmental samples to be sonicated and even with a weak selective pressure the plasmid will be maintained and expressed all with minimal manipulation and retention of the sample in its indigenous environment.

Introduction

Following the low transformation rates found using alternate plasmids it was decided to test one of the main proposed benefits of UGT: that even with a low transfer rate selective pressure would ensure that the transformed cells would out compete the remainder of the community and become dominant enabling them to become increasingly effective over a few generations.

Plasmid pBAV1K was used as, whilst the transfer rate was previously low, the strong GFP expression allows for ease of monitoring the samples during the incubation. A preliminary experiment to assess suitable conditions and time periods showed despite no initial transformants being seen on selective plates, increased transformant counts were seen after 2 days incubation with antibiotic selective pressure which provided a baseline for the parameters of this experiment. It should again be noted that due to the ageing of the first sonotrode and the continued delays in sourcing and manufacture of the second generation sonotrode these experiments were performed using shortened exposures with the first generation sonotrode.

Materials and Method

The direct immersion procedure with 20 ml of *P. putida* UWC1, 1 ml 1M $CaCl_2$, 20 µl of plasmid pBAV1K was used with 1.5 minute exposure at 27.8 kHz. Following exposure samples were incubated for 2 h at 27 °C. After incubation 5 replicates of each sample were inoculated with Kanamycin Sulphate to concentrations of 50, 20, 10 and 0 µg/ml, initial samples were taken at this point, day 0, then at day 2, 7 and 14, these were spread on LB plates supplemented by 50 µg/ml of Kanamycin to quantify the number of cells containing the plasmid and suitably diluted and plated on LB plates to quantify total cell counts.

Results

No transformants were seen on 50 µg/ml Kanamycin agar plates in any of the samples at the day zero time point (with two hours incubation following sonication) and all samples produced circa 10^9 colonies on non-selective agar plates. The 50 µg/ml samples and negative controls saw a sharp drop in total CFUs as the antibiotic killed a large proportion of the cells within the first two days and by day 14 no viable cells remained in the negative controls with no ultrasound and with no plasmid. However by 7 days the 50 µg/ml test sample the number of colonies on the selective plate equalled those on the non-selective reaching parity and maintained this level until the day 14 sample (see Figures 34 and 35).

For the lower doses of Kanamycin, that were below the lethal threshold of the cells, the replication of CFUs on the non-selective media was not noticeably restricted reaching a peak of circa 10^{12} CFUs for 20, 10 and 0 µg/ml of Kanamycin. Whilst plasmid containing CFUs increased over the first 2 days for 10 and 20 µg/ml before plateauing at an average of 10 and 1000 CFUs respectively, no expression was seen on any of the selective plates for the samples inoculated with 0 µg/ml of Kanamycin.

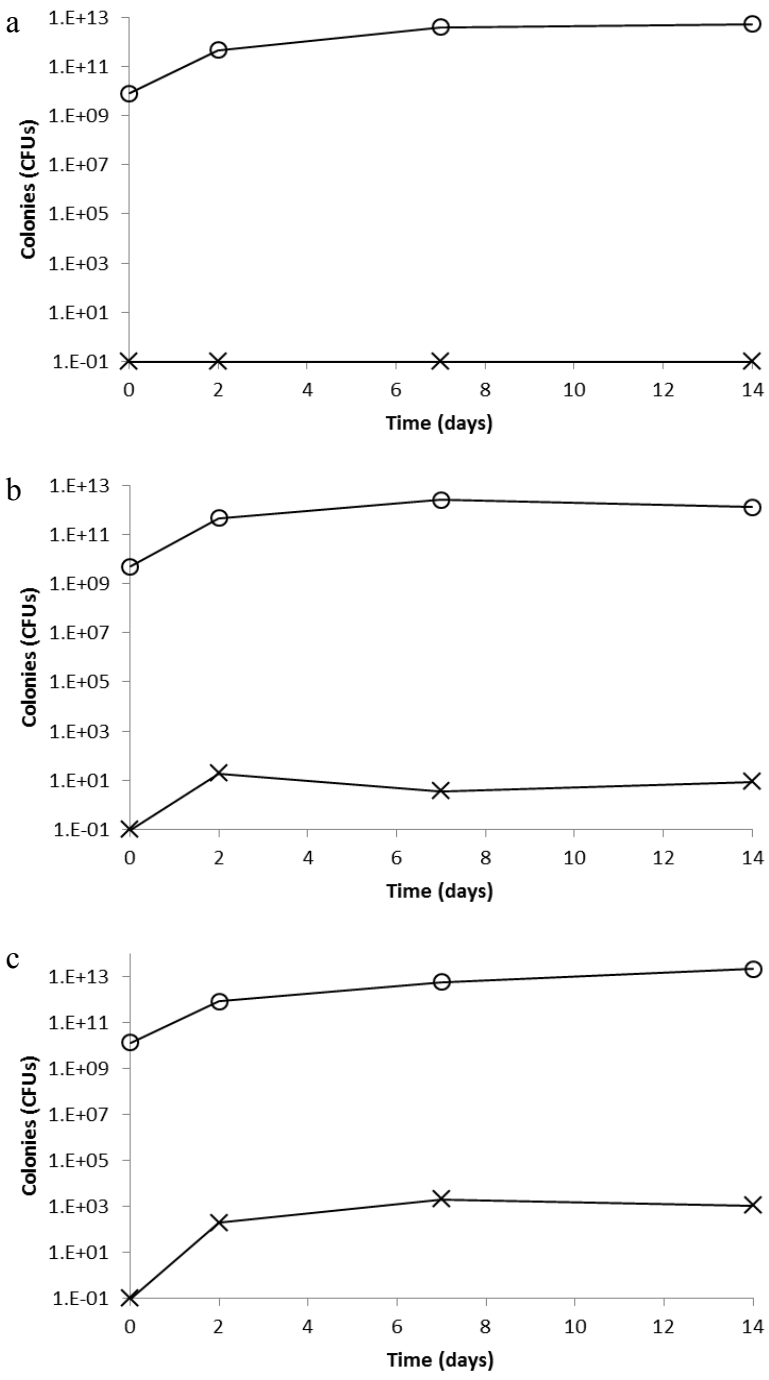

Figure 34: pBAV1K Expressing CFUs (x) v non-transformed CFUs (o) with Antibiotic Selective Pressure over Time, showing the effect of selective antibiotic pressure on transformed and untransformed cells a) 0 µg/ml Kanamycin, b) 10 µg/ml, c) 20 µg/ml, d) 50 µg/ml, e) No Ultrasound (50 µg/ml), f) No Plasmid (50 µg/ml) Average of 5 biological replicates. No error bars shown as the log scale renders them illegible. (cont. Figure 35)

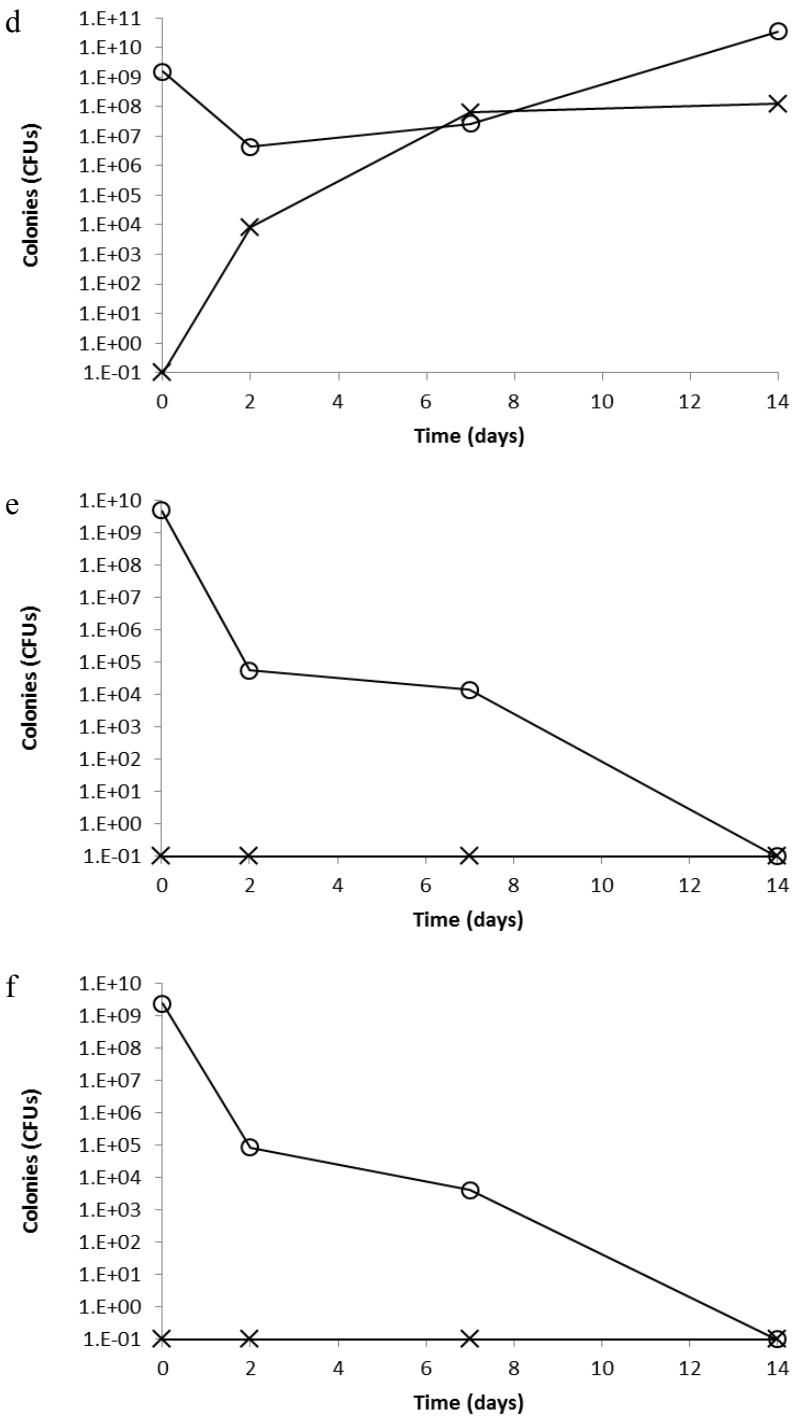

Figure 35: pBAV1K Expressing CFUs (x) v non-transformed CFUs (o) with Antibiotic Selective Pressure over Time, showing the effect of selective antibiotic pressure on transformed and untransformed cells (cont. from Figure 34) a) 0 µg/ml Kanamycin, b) 10 µg/ml, c) 20 µg/ml, d) 50 µg/ml, e) No Ultrasound (50 µg/ml), f) No Plasmid (50 µg/ml) Average of 5 biological replicates. No error bars shown as the log scale renders them illegible.

Discussion

Selective pressure is shown to quickly force the plasmid carrying bacteria to become dominant when the antibiotic is strong enough to eliminate non-resistant cells with all the cells expressing the gene after 7 days, followed by some recovery of the total cell count by day 14, full recovery would be expected after further incubation to comparable levels with the other less potent Kanamycin concentrations. In the samples where there was no plasmid or the cell/plasmid suspension was not exposed to ultrasound, no transformants were seen and therefore unable to become dominant hence no colonies were produced throughout from any of the replicates for any of the negative controls, meanwhile the lethal dose of Kanamycin prevented any replication of non-transformed cells leading to their death and complete extermination by 14 days.

Where there is no selective pressure the plasmid is not retained and no Kanamycin resistant or GFP colonies were seen on the selective plates and the total colony count, once the sample had recovered from sonication, remained in the stationary phase.

Of most interest are the mid-range samples were the selective pressure has been sufficient for some transformants to be maintained without killing or restricting the remainder of the cells. For both 10 and 20 μg/ml concentrations the plasmid containing cells multiplied to a baseline level reached on day 2, the relative volumes of plasmid versus no plasmid cells then remained roughly constant for the remainder of the experiment. These results show, that even for a reduced selective pressure, a percentage of the community will retain the plasmid whilst minimising the negative effect on the total cell numbers.

Within two days it is shown that the presence of a selective pressure allows cells that have been transformed by UGT to express the GFP genes on the plasmid, remain viable and replicate. This clearly demonstrates the benefit of this procedure's direct application, as following sonication and the addition of the selective pressure no further manipulation was required and the cells were able to survive and reproduce satisfactorily in the media that they were prepared and sonicated in; unlike the requirements for other transfer methods.

Conclusions and Progression

Even with low transfer efficiency UGT is able to provide a base number of transformants that within 2 days can dominate a community with a strong selective pressure and with lower selective pressure the plasmid containing cells can replicate and result in a notable proportion of the cells being resistance and expressing the selected gene which will be ideal for environmental applications as the degradation ability conferred is unlikely to provide a dramatic advantage and hence whilst enabling and improving the selected degradation pathway will not seismically change the community, thus providing a balance between the potential impacts and the benefits of adjusting the genetics of the community.

4.4: Applying UGT to Coke Oven Effluent Biosludge and Sampling by Laser Micro-dissector

Abstract

UGT was applied to a biosludge sample from the coke oven effluent treatment plant at Tata Steel Scunthorpe. Plasmid pBAV1K was used, containing a GFP gene and Kanamycin resistance gene for identification and selection respectively. Raw data suggested that transfer had occurred, as an increase in the number of fluorescent particles, suggesting pBAV1K expression, in the treated samples with selective pressure being observed however statistical analysis found this non-significant. Attempts to isolate single transformed cells, identified by fluorescence microscopy, from the biosludge using a laser microdissector and extracting the DNA for analysis proved fruitless. This led to the conclusion that UGT had not occurred at sufficient or consistent levels to be proven conclusively in this complex community of biosludge. The suggested limiting factors are the solid particles in the biosludge interfering with the cell/plasmid/cavitation interactions prevent adequate sonoporation for transfer to occur. Improving the transfer rate and consistency is key to any future complex community trials.

Introduction

Following the success of selective antibiotic pressure enabling transformants to become dominate in *P. putida* UWC1 grown in LB broth and receipt of a fresh sample of biosludge from a biological treatment tank for coke oven effluent at Tata Steel's Scunthorpe plant it was decided to attempt UGT with this sample using the

pBAV1K plasmid. Ideally further development of the procedure prior to this point would have been performed however the recent delivery of a fresh biosludge sample dictated the schedule. The selective Kanamycin pressure would then be applied to encourage transformants to express the GFP gene and out compete non-transformed cells. Following filtration to collect the bacteria the number of cells expressing GFP would be assessed under a fluorescence microscope with a FITC (fluorescein isothiocyanate) filter and any increase in fluorescence following sonication is then identified. Analysis by microscopy was chosen over selective plates as it is expected that many of the bacteria in the biosludge sample would be unculturable and hence a representative data set would not be produced and any unculturable transformants would be missed.

Once the cells numbers were quantified it was attempted to isolate single transformants using a laser micro-dissector, this apparatus uses special polymer slides which allow for the slide first to be explored under the microscope then using a computer controlled laser cut through round the selected cell which is then ejected into a tube for collection. These cells were then lysed with the DNA extracted and PCR performed to attempt to confirm the transformed plasmid within.

This aims to provide clear confirmation of UGT of a marker plasmid into an environmental sample and give a comprehensive proof of the mechanism behind the technique.

Materials and Method

Biosludge was sampled from the biological treatment pond for coke oven effluent treatment at Tata Steels' Scunthorpe plant (sample date: 13/7/12). This was stored in cold room conditions (~4°C) until use. The sample was separated into 20 ml aliquots to which 1 ml of 1M $CaCl_2$ was added and 25 µl of plasmid pBAV1K. The samples were sonicated for 1.5 minutes at 27.8 kHz then allowed to recover for two hours. Negative controls with no ultrasound and with no plasmids were also performed. Following the recovery incubation the samples were dosed with Kanamycin Sulphate to concentrations of 50, 20, 10 and 0 µg/ml to encourage gene expression and provide selective pressure.

For microscopy a sub-sample was taken from each tube and serially diluted a thousand-fold with sterile UHQ, 1 ml of this was pipetted into a clean and sterile

glass funnel and drawn by vacuum through a 0.22µm filter, black polycarbonate Ø 25 mm (Osmonics Inc. USA), the funnel was washed twice with UHQ through the filter. The filter was then removed and placed on a glass slide and a few drops of UHQ added prior to the coverslip being lowered over the sample.

The sample was examined using a Zeiss Axioplan 2 epifluorescence microscope with a mercury fluorescence lamp and imaged with a colour CCD camera, each sample had 20 fields imaged with both bright field images and fluorescent images using an FITC filter. Each image was examined and had the number of fluorescent cells counted for analysis.

To isolate cells that carried the plasmid collaboration with Dr Peter March at the Bioimaging Facility in the Faculty of Life Sciences at the University of Manchester was undertaken. This enabled use of the facilities Laser Microdissector apparatus: PALM Microbeam (P.A.L.M. Microlaser Technologies AG, Germany), this apparatus combines a high precision laser (337 nm pulse beam UV laser) connected to a Zeiss Axiovert 200 microscope with a computer controlled specimen stage, PALM Robostage, with sub-micrometre sensitivity and the associated PALM CapMover sample collection arm.

The sample was spread on a PEN polymer microscope slide (Life Technologies, UK) and allowed to dry prior to storage; preparation was performed in the Sheffield laboratory and transferred to Manchester the following day for the procedure. The PEN slides enable the laser to quickly and accurately cut through the surface to eject the cells into the collection cap.

Samples slides were examined for fluorescing cells with 40x magnification and FITC filter, once a suitable cell was located the PALM@Robo v2.2 software was used to select the cell location, cut around the cell and eject it into the overhanging cap. This was then sealed and returned to Sheffield for extraction, purification and analysis.

To harvest the DNA from the ejected cells a Repli-g Mini kit (QIAGEN, UK) was used, following the ‘Protocol: Amplification of Genomic DNA from Blood or Cells’: 3µl of PBS (phosphate buffered saline) and 3.5 µl of buffer D2 were added to the cap and re-sealed, this was vortexed then centrifuged (4000 rpm using a benchtop

centrifuge) twice, the recommended procedure was then followed. Once extracted PCR was performed using 0.3 µl Dream Taq (Fermentas), 2 µl 5 mM dNTPs, 5 µl PCR buffer (Fermentas), 2 µl forward primer (GFP_ADP1_for_EBglII), 2 µl reverse primer (GFP_ADP1_rev_EBglII) and 38.5 µl nuclease free dH_2O.

PCR was performed using S1000 Thermocycler (Bio-Rad, UK) with the following cycle:

- 95 °C – 5 min
- (95 °C – 1 min
- 54 °C – 1 min
- 72 °C – 1 min) repeat 34 times
- 72 °C – 5 min
- Maintain at 12 °C

The resultant sample was purified using QIAquick PCR Purification kit (QIAGEN, UK) following the manufacturer recommended procedure and sent for sequencing to assess the products.

Results

Initial analysis of microscope images showed fluorescent particles across the samples with an increasing amount for the kanamycin spiked samples to provide selective pressure.

This is shown in these images (see Figures 36 and 37) from samples taken on Day 22 of incubation with the respective selective antibiotic concentrations show the clumps of solid material within the biosludge suspension with minimal numbers of unattached micro-organisms. The fluorescent particles seen in the FITC images can be correlated with the bright field images and identified; none of these were identified as rod shaped bacteria, however a few are potentially coccoidal and within a realistic size range.

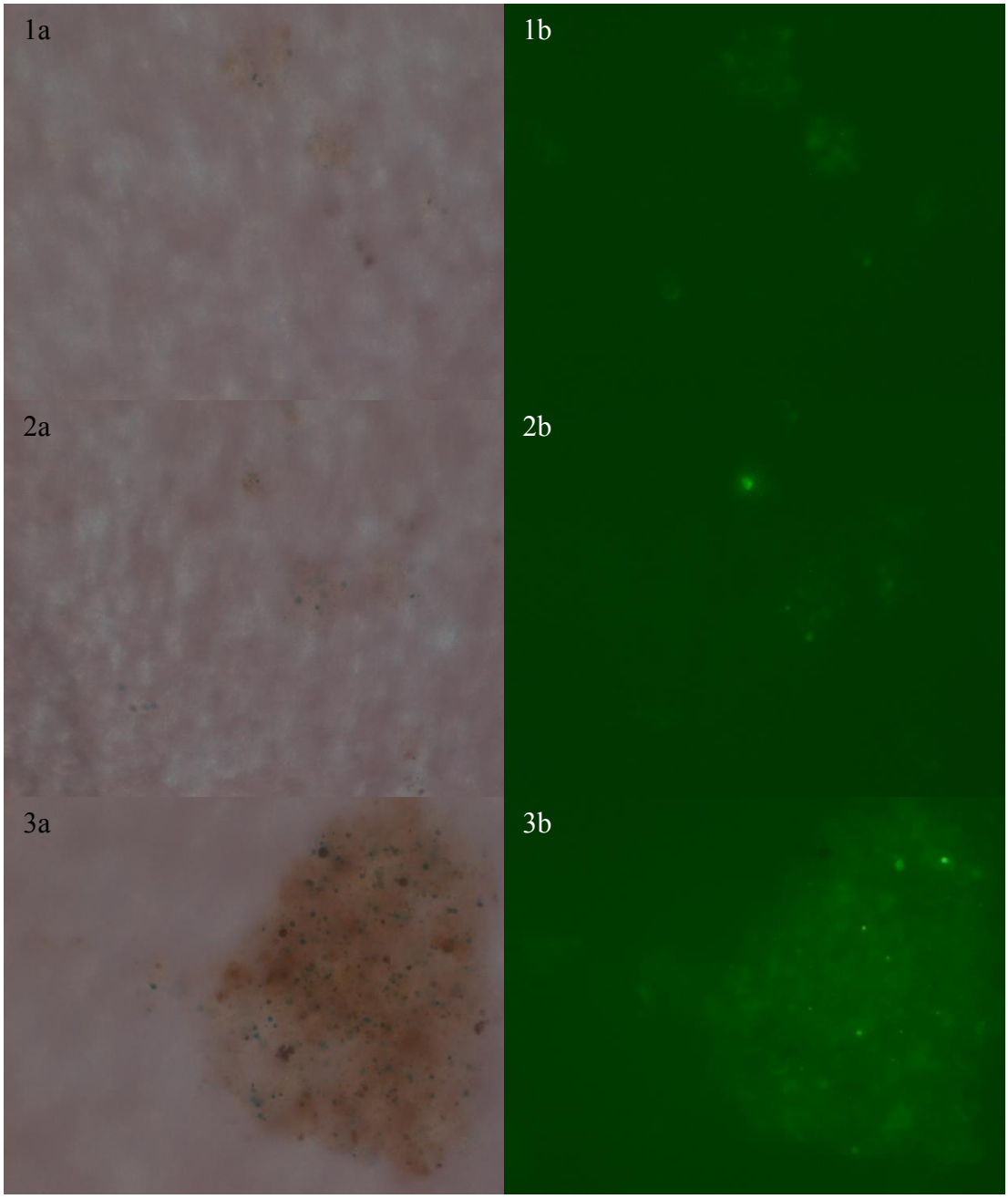

Figure 36: Microscopy of Biosludge with UGT of pBAV1K, incubated with Kanamycin selection, bright fluorescent spots on the FITC filtered image show potential transformants or naturally fluorescent particles 1) 0 µg/ml Km, 2) 10 µg/ml Km, 3) 20 µg/ml Km, a) bright field images, b) UV images with FITC filter, 1 µl of sample collected by vacuum on 0.22 µm black polycarbonate filter disc

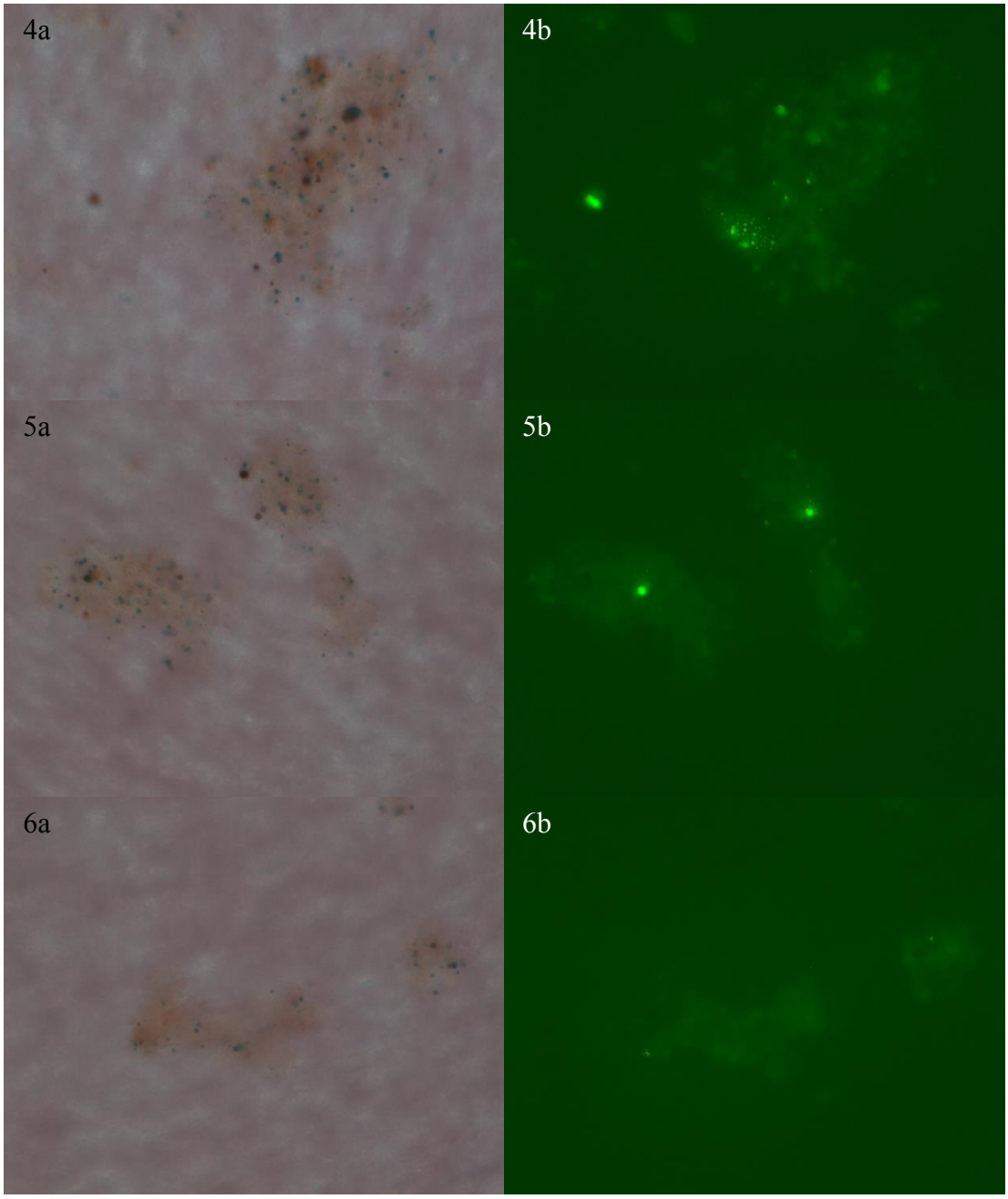

Figure 37: Microscopy of Biosludge with UGT of pBAV1K, incubated with Kanamycin selection, bright fluorescent spots on the FITC filtered image show potential transformants or naturally fluorescent particles 4) 50 µg/ml Km, 5) No Plasmid with 50 µg/ml Km, 6) No Ultrasound with 50 µg/ml Km, a) bright field images, b) UV images with FITC filter, 1 µl of sample collected by vacuum on 0.22 µm black polycarbonate filter disc

The count of fluorescent particles are highest for the 20 and 50 µg/ml samples showing a peak after 15 and 22 days however there is wide variation between samples (see Figure 38) shown by the large error bars of one standard error. Analysis by one-way ANOVA (SPSS, IBM, USA) showed no statistical significance between the test samples and the negative controls ($p>0.05$) for any of the time points tested.

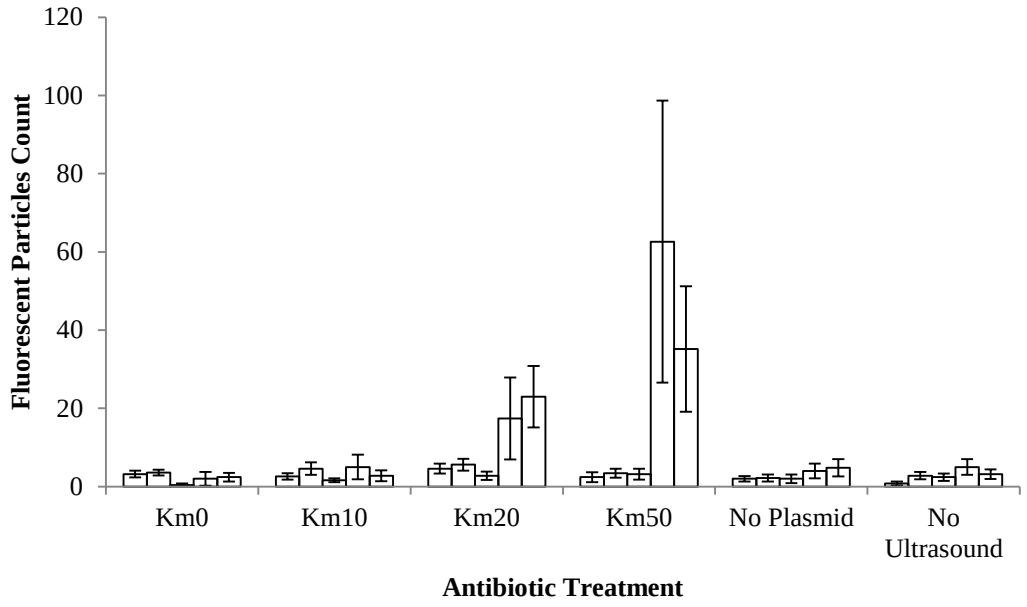

Figure 38: Particles fluorescing under UV light with FITC filter after 0, 3, 8, 15 and 22 days incubation 5 replicates for each sample, 10 fields imaged of each replicate, counts averaged and 1 S.E. shown

Following these inconclusive results, it was attempted to isolate single cells with the laser microdissector and extract the DNA to investigate whether the plasmid was present.

Fluorescent particles were located (Figure 39), selected, cut and ejected into the collection cap, once PCR was performed and agarose gel was performed however no DNA was found using pBAV1K coded primers was identified.

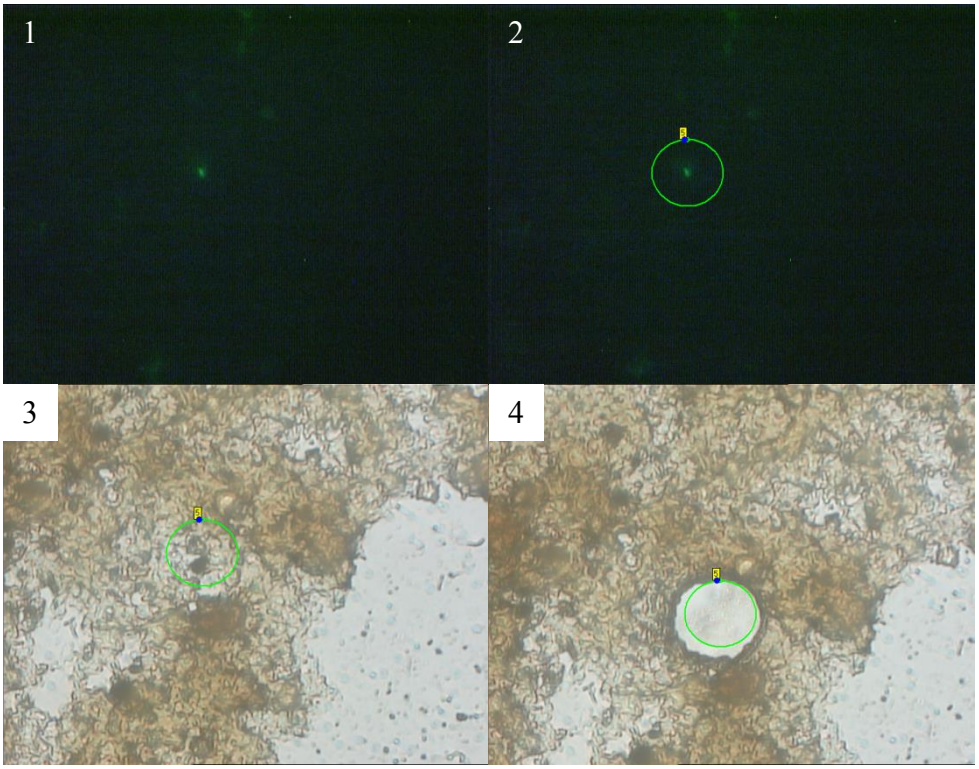

Figure 39: Laser Micro-dissector step-by-step images 1) locate fluorescent particle with FITC filter, 2) select with PALM@Robo v2.2 software 3) bright field image 4) bright field image following laser cutting

Discussion

The initial data and microscope images suggested that the plasmid had been successfully transferred into the biosludge samples and then reproduced under selective pressure; 10-fold increases were seen over negative controls however despite a reasonable sample set the range of values across the replicates was so wide that a few samples skewed the raw data and following statistical analysis these were shown to be non-significant at a $p>0.05$ threshold. Following results of the laser microdissector experiment, PCR amplification and characterisation, transfer of this plasmid into the biosludge bacteria has not been demonstrated and is unlikely to be proven without modification to this experimental set-up.

Previous experimental work with *P. putida* UWC1 monocultures grown in LB broth also showed variation in transfer rates between samples however the variations were statistically significant against the controls unlike in this experiment. On this basis with further refinements to the technique to reduce variability between samples improving the uniformity of the transfer to a statistically significant level is hopeful.

During sonication agitation of the sample was seen, however this was not to the extent that was observed when using a bacterial suspension in LB broth or when water control preparations were sonicated. This is most likely due to a damping effect caused by the solid sludge particles in the sample absorbing the ultrasonic waves and blocking any cavitation that might occur. This will drastically decrease the number of cell/plasmid/cavitation interactions which are key to UGT. Similarly the solid debris in the sample may attach to the plasmid immobilising it or the bacteria may be partially embedded in the debris reducing their receptive surface area. All these factors will reduce the triad of interaction that has been shown in this work to be the driver behind UGT. If exposure times are increased for future work overheating of the sample from absorbance by the biosludge should be controlled (potentially using a cooling water jacket) and destruction of the aggregates and dispersion of biofilms within the biosludge should also be monitored as this may affect the existing abilities of the biosludge to degrade contaminants. Alternatively, temporary isolation of the bacterial community with filtration to remove solids prior to sonication and then re-introduction into the environment could be considered, however this markedly increases complexity and raised culturability issues of maintaining the community *ex situ*.

As fluorescence was seen under the microscope with the mercury lamp and FITC filter in all the samples including the negative controls (see Figures 36 & 37), it can be concluded that there is naturally occurring bio-fluorescence in the sludge, whether from bacteria, cyanobacteria of other single cellular micro-organisms it is unknown. However, under incubation these organisms did replicate across the samples with the few samples seeing large clusters formed. It should also be noted that larger objects were seen under with the mercury lamp that corresponded with debris seen in the bright field images, however these were not included in any counts as they were too large to be bacterial.

Using the laser microdissector was a novel piece of apparatus and whilst the fluorescent cells were cut round and ejected, there is uncertainty as to whether they were successfully caught in the collection cap. Considering only a few cells were ejected toward each cap the raw biological material for DNA extraction is minimal and can easily be damaged during preparation for PCR. The PCR procedure is fairly

robust for replicating DNA once extracted however if the there is a failure in the preparation and cell collection stage this will not be identified until after the PCR.

Conclusions and Progression

Whilst no transfer of the plasmid into the bacteria indigenous to the biosludge has been shown to a statistically significant level the results achieved suggest with improvements to the uniformity of the sonication and resultant transfer rate UGT may be shown to be suitable for transforming such complex communities as the biosludge used here.

The current limitations to successful application to biosludge or a similar community are the interactions between the ultrasound and any solids in the sample causing damping or reduction of the effectiveness of the cavitation effect. Secondly the current lack of successfully transformed species either in monocultures or as simple communities reduces confidence in the ability of UGT to transfer to a range of species following direct application to a complex community.

To improve operation and potential success with the laser microdissector: practice with control samples to eject, collect and extract known DNA is recommended thus reducing the wide margin for error found here.

Prior to further work using the biosludge it is recommended to obtain an alternative fluorescent gene that is less likely to be naturally occurring and hence be more clearly distinguished, or alternatively introduce a degrading gene and monitor the degradation relative to any controls if possible within the timeframe of this project or to be undertaken in the future.

4.5: UGT of pBAV1K (GFP marker) into Multiple Species and pMTC9 (mCherry marker) into River Water

Abstract

UGT was performed on 3 species of bacteria: *P. putida* UWC1, *E. coli* S17 and *S. putrefaciens*, as both monocultures and mixtures of the 3. Colonies on selective media following UGT were only seen from *P. putida* monocultures. Similarly when attempted on concentrated river water samples no transformants were identified by microscopy, whilst positive controls were successfully transformed.

Whilst this does not disprove the possibility of UGT in a range of species it shows one size does not fit all for the UGT parameters, this could however be used to targeting of species within a mixture if adjustments are made. The UGT procedure remains robust for transfers using *P. putida* UWC1 and as such continued work with this strain remains an appropriate target.

Introduction

An artificial mix of species grown under laboratory conditions in an attempt to minimise the potential unknowns in the procedure, following the difficulties encountered using the biosludge. Three laboratory culturable species were chosen each naturally not resistant to Kanamycin, including the previously used *P. putida* UWC1, with plasmid pBBR1MCS-2, to provide continuity to compare with previous work. The other species were *E. coli* S17 and *Shewanella putrefaciens*; the *E. coli* strain was selected as a commonly used lab strain, whilst the *Shewanella* was a different alternative as a marine anaerobe to investigate whether the transfer could work in this variety of species.

This was followed by an attempt to transfer plasmid into the bacterial community within a natural water sample; a reservoir water sample was chosen as the solid particles found in the biosludge were thought to be a potential hindrance to cavitation and its effect on the cells. The sample was taken from Damflask Reservoir which is a compensation reservoir on the edge of the peak district, local to the university. The organic component in the sample, including the indigenous micro-organisms, was very low so the sample was concentrated by tangential flow filtration to improve the likelihood to of cell/plasmid/cavitation interactions. Due to the prevalence of organic material which shows fluorescence similar to that seen from GFP in pBAV1K when using an FITC filter; it was decided to use a plasmid containing the mCherry gene: pMTC9 (Cairns et al., 1997, Jappelli and Brenner, 1998), which also contains ampicillin antibiotic resistance.

Materials and Method

Multiple species UGT: *P. putida* UWC1, *E. coli* S17 and *Shewanella putrefaciens* were selected, these were grown separately overnight in LB broth at 30 °C, 37 °C and 30 °C respectively. OD600 was measured using a Biochrom WPA Biowave

S2100 Spectrophotometer (Biochrom, UK) prior to use for reference during later analysis.

Samples were prepared with 5 replicates containing an equal mixture of portions of each species totalling 20 ml, 5 replicates of 20 ml of each individual species, with 3 replicates for negative controls of the 3 species mixture with no plasmid and 3 with no ultrasound and 3 replicates of each single species sample also with no ultrasound and 3 with no plasmid. 20 µl of plasmid pBBR1MCS-2 was added to each selected sample and the requisite samples were sonicated at 30 kHz for 3 minutes with the second generation ultrasonic horn. Samples were allowed to recover for 2 hours at 30 °C before plating on LB media plates supplemented with 50 µg/ml of Kanamycin Sulphate. Plates were examined and colonies counted after overnight incubation at 30 °C and re-examined after 48 hours.

River Water UGT: 10 litres of lake water was collected from the surface of Damflask Reservoir. This was concentrated by tangential flow filtration which cycles the sample through a manifold containing a 0.22 µm filter mesh which enabled a fraction of the water content to be reduced every cycle whilst the micro-particles were retained in the sample and hence concentrated incrementally. The sample was concentrated by this method to 200 ml (i.e. 50 times the concentration). No further reduction was possible due to the volume requirements necessary for the transfer. The filtered water would be retained with the intention of using it to re-dilute the sample prior to reintroduction into the environment following a successful treatment.

The plasmid used was pMTC9. This was prepared form plasmid stocks and inserted into *E. coli* DH5α by heat shock; strongly expressing transformants were grown in LB broth with Ampicillin at 100µg/ml. Plasmid extraction was performed using a Miniprep Kit (Qiagen, UK) and confirmed by agarose gel electrophoresis.

20 µl of plasmid was added to 20 ml of concentrated water sample and sonicated for 2.5 minutes at 27.5 kHz; samples were then allowed to recover for 2 hours at room temperature, 100 µg/ml Ampicillin selective pressure was then added to the test samples. Negative controls with no plasmid or no ultrasound were also performed as well as positive controls using *P. putida* UWC1 and pMTC9 or pBAV1K.

The samples were imaged using a Zeiss Axioplan 2 epifluorescence microscope with a mercury fluorescence lamp and imaged with a colour CCD camera, each sample had 20 fields imaged with both bright field images and fluorescent images using a Cy3 filter. Each image was examined and had the number of fluorescent cells counted for analysis.

Results

Multiple species UGT: No transformants were seen on any of the plates from the 3 mixed species samples, and for the single species samples transformants were only seen on the *P. putida* UWC1 plates at a lower efficiency than usual. No transformants were seen on the selective plates for either of the *E. coli* S17 or *S. putrefaciens* monoculture samples. Colonies were seen at a usual $10^6 – 10^8$ range of magnitude on non-selective media for the monocultures and the mixed samples were dominated by the faster growing *P. putida* UWC1. It should be noted that the OD600 for *S. putrefaciens* was ~0.304, whilst *P. putida* and *E. coli* were 0.68 and 0.570 respectively.

River Water UGT:

Red fluorescence was seen in the test samples but was found to correspond to naturally fluorescing micro-organisms which were also present in the control samples (see Figure 40). No bacterial transformants could be successfully identified. The positive control introducing pMTC9 into *P. putida* UWC1 was successful at a rate similar to previous work.

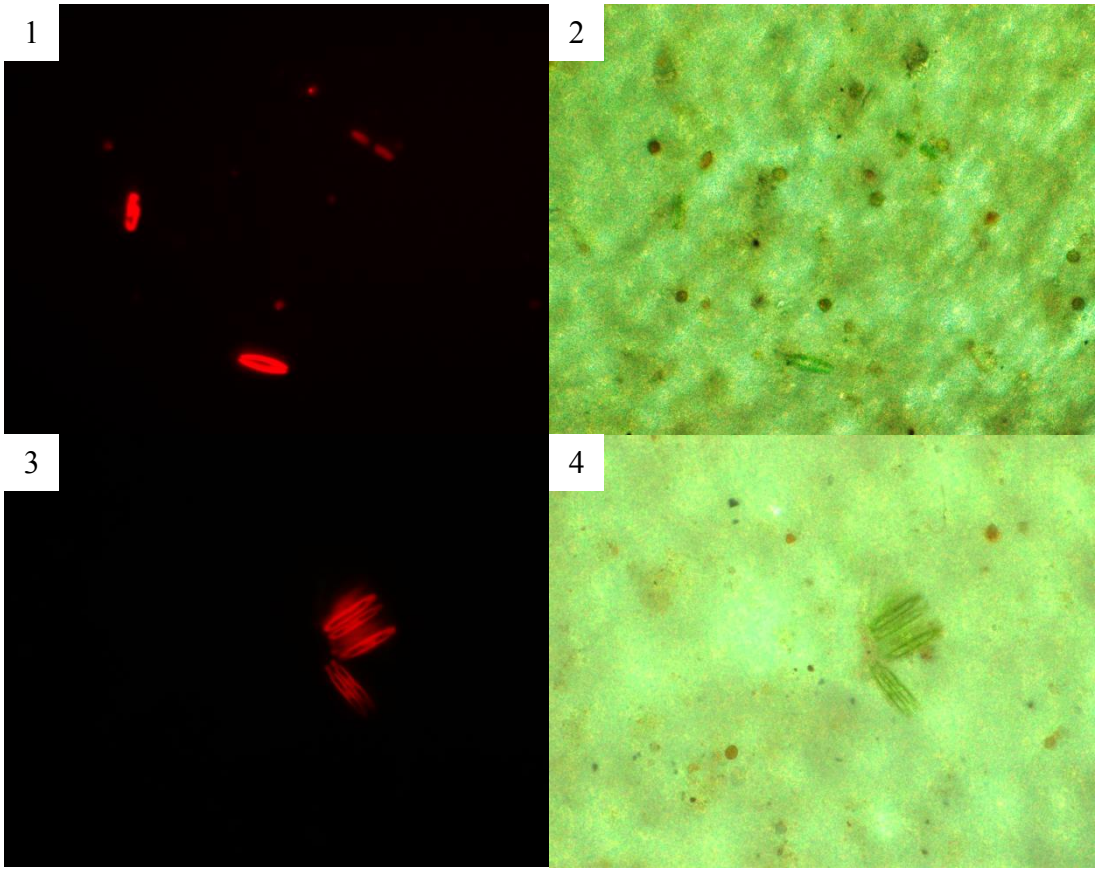

Figure 40: River Water Samples following UGT with pMTC9 1 & 2) pMTC9, 3 & 4) No plasmid, 1 & 3) Cy3 filter, 2 & 4) Bright field image

Discussion

Multiple species UGT: The production of transformants from only the *P. putida* monocultures is a setback and whilst the absence of proof is not a proof of absence a low transfer rate under relatively amenable conditions reduces the confidence for developments despite the potential for transformants to dominate under selective pressure. It should however be noted that no $CaCl_2$ was added to the samples prior to sonication which is shown to have a beneficial effect on the transfer rate as the water sample was expected to naturally contain suitable $CaCl_2$ levels, however this was not chemically characterised.

The lack of expression in the other species could be down to plasmid incompatibility, which is possibly true for the *Shewanella* strain but highly unlikely in the *E. coli* strain as the plasmid was grown and extracted from *E. coli* DH5α, a close relative of the S17 strain used. There may also be added stress to the *Shewanella* as it was not maintained under its preferred anaerobic conditions

however it had previously been incubated on agar plates in the presence of atmospheric oxygen.

River Water UGT: Again attempted transformation of an environmental sample has been unsuccessful whilst the positive controls confirmed the plasmid and sonication were effective under these parameters. Despite concentrating the river water to increase the bacterial concentration it was still markedly lower than that used for the single species control, which may account for the lack of transfer. Although a broad range plasmid was used the plasmid compatibility with the indigenous species may result in the plasmid not being expressed, as such characterising the indigenous species prior to transfer and then selecting a suitable plasmid could lead to an improvement. Unfortunately due to time constraints as this was the last experiment these adjustments could not be performed, as such whilst UGT has been successful in single species transfer into a multi-species natural community has yet to be achieved.

This continued inability to introduce plasmids to environmental samples is frustrating and has yet to be solved however the suggested causes are the reduced nutrient conditions leading to reduced expression of unnecessary genes and reduced ability for the cell to recover from the cavitation damaged caused by UGT. The cell/plasmid/cavitation interaction that is identified as the mediator is also reduced in environmental samples as the density of target cells is much reduced and there is often other particulate matter that will interfere with the desired cell/plasmid/cavitation interactions. The environmental samples will also have differing salt and pH conditions to the model LB broth with 50 mM $CaCl_2$ that is used for single species transfer; this will affect the cell structure of the bacteria specifically the cell walls and plasmid binding and uptake and subsequent cell recovery. For future work comprehensive chemical and biological assessments are suggested, especially with respect to salt conditions, biomass and the bacterial portion of this.

Conclusions and Progression

P. putida UWC1 remains a robust and reproducible target for UGT however transfer to other species and environmental samples has been less successful, the physical nature of cavitation being the UGT transfer mechanism suggests that there should be

a wide range of recipient species, as the damage to the cell walls from microbubble cavitation should damage all cells to an extent, requiring only an adjust of exposure to maximise transfer. There do not seem to be any fundamental reasons that UGT would not be successful and with adjustment of parameters, especially cell and plasmid concentrations, it is expected that UGT into an environmental community will be achieved.

The use of selective pressure over an incubation period can also aid demonstration of transformation as even if only a few cells are transformed they will be able to reproduce and spread the gene whilst the selective pressure benefits them.

5: Delivery of High Molecular Weight Chemicals into Bacteria

Whilst the primary goal of this work is to introduce pollutant degrading genes into bacteria, an alternative use for ultrasonic transfer was considered; namely the insertion of chemicals into bacteria, potentially for the application of drugs or other medical treatments. This also allowed for further investigation into the mechanism of transfer and the limits or possibilities to improve the method.

A range of sized marker chemicals are investigated from short chain polymers to a compound of similar size to a small plasmid and analysis is performed with fluorescence microscopy of labelled compounds.

Abstract

Two different sized fluorescent labelled dextran molecules (70,000 & 2,000,000 MW) have been successfully delivered into *P. putida* UWC1 using the sonotrode and procedure that has been developed in this work. Transformants were identified by fluorescence microscopy and efficiency was similar to that found when transferring plasmids, in the order of 10^{-6} – 10^{-7}. This delivery paves the way for the potential transport of macro-molecules, such as drugs or high molecular weight chemicals, using bacteria and potentially enabling delivery of bioparts and nanomaterials for synthetic biology to targeted locations in an organism. Microscopic imaging has also detected effects on the cells' morphology following sonication despite a recovery period and further research is needed to investigate if this is a long term effect.

Introduction

Ultrasonic transfer is a physical technique to deliver macro-molecules such as DNA; it is hypothesised that other macro-molecules such as carbohydrates, proteins, drugs and nanomaterials could also be delivered into cells, which the cell transportation system (e.g. diffusion and active transportation) is unable uptake. This entailed investigating macro-molecular chemical delivery into the bacterial cells, specifically fluorescent marker chemicals of differing sizes that could be enumerated by microscopic imaging of transformants. It was hypothesised that the 'smaller' macro-molecules would be transferred more easily as they would pass through the pores created by sonication more easily, as it has been seen in previous work with different plasmids that there was variation in transfer efficiency with respect to size between plasmids. The selected macro-molecules were two fluorescent dextran molecules,

one of 70,000 MW and the other 2,000,000 MW; chosen as a 'small' macro-molecule and a macro-molecule close to the size of a small plasmid respectively. The fluorescent markers were selected, from those offered by the manufacturer, to be easily identifiable using the laboratory fluorescent microscope. The charge of the molecules must also be noted as the current experimental set up is optimised with $CaCl_2$ to mitigate the high surface negative charge on the plasmids from the phosphate groups. Negatively charged macro-molecules were chosen for use with $CaCl_2$ or neutrally charged macro-molecules to minimise the experimental deviation from plasmid transfer.

Materials and Method

Two large molecules were selected for transfer; both were labelled dextrans, which have a backbone of hydrophilic polysaccharide (Life Co., UK). A Texas red (Excitation/Emission wavelength: 595/615) dextran of 70,000 MW, with 4 moles of dye per mole of dextran. Fluorescein (Ex/Em wavelength: 494/521) tagged 2,000,000 MW dextran, 120 moles of dye per mole of dextran.

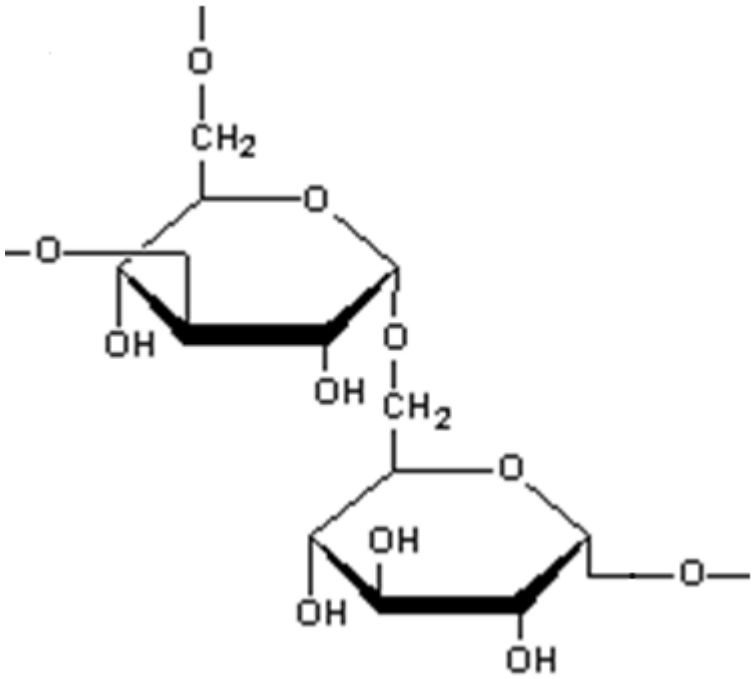

Figure 41: Backbone Structure of Dextran Molecule modified from Sigma Aldrich catalogue

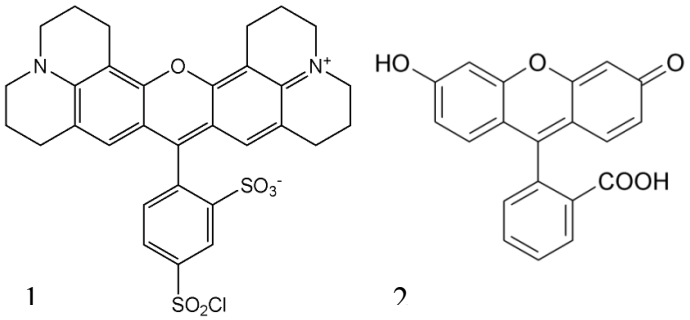

Figure 42: 1) Texas Red Dye Molecule, 2) Fluorescein Molecule modified from Life Technologies catalogue

The transfer procedure was performed using the direct immersion method, sonicating 20 ml of overnight growth of *P. putida* UWC1 at 30 kHz for 3 minutes. 5 biological replicates of each molecule were performed.1 mg of Texas red dextran or 0.1 mg of fluorescein dextran was suspended in 1 ml of sterile water and added to the samples and briefly mixed prior to sonication (final concentrations of 714 and 2.5 nM/L respectively). Negative controls with either no ultrasound or dextran were also performed, as were positive controls using the pBAV1K plasmid (~100 ng of DNA). Following sonication samples were incubated for two hours for recovery at 30 °C.

For imaging the samples were pelleted by centrifugation then washed with PBS, this was repeated in triplicate, to remove any trace of any marker dye that had not been up taken. The sample was collected by vacuum filtration onto a 0.22µm filter, black polycarbonate Ø 25 mm (Osmonics Inc. USA). The sample was examined using a Zeiss Axioplan 2 epifluorescence microscope with a mercury fluorescence lamp and imaged with a colour CCD camera, each sample had 20 fields imaged with both bright field images and fluorescent images using an FITC filter. Each image was examined and had the number of fluorescent cells counted for analysis.

Results

Transformants were seen across all test samples for both the Texas red and fluorescein labelled dextrans, however only a few cells were strongly fluorescing for the image fields sampled, whilst there were hundreds/thousands of untransformed cells (see Figures 43-45). No transformed cells were seen on the sampled image fields on negative control slides or following a thorough search across the whole slide. Positive controls with the GFP expressing plasmid, pBAV1K, also produced transformants at a third of the rate of fluorescein labelled dextran.

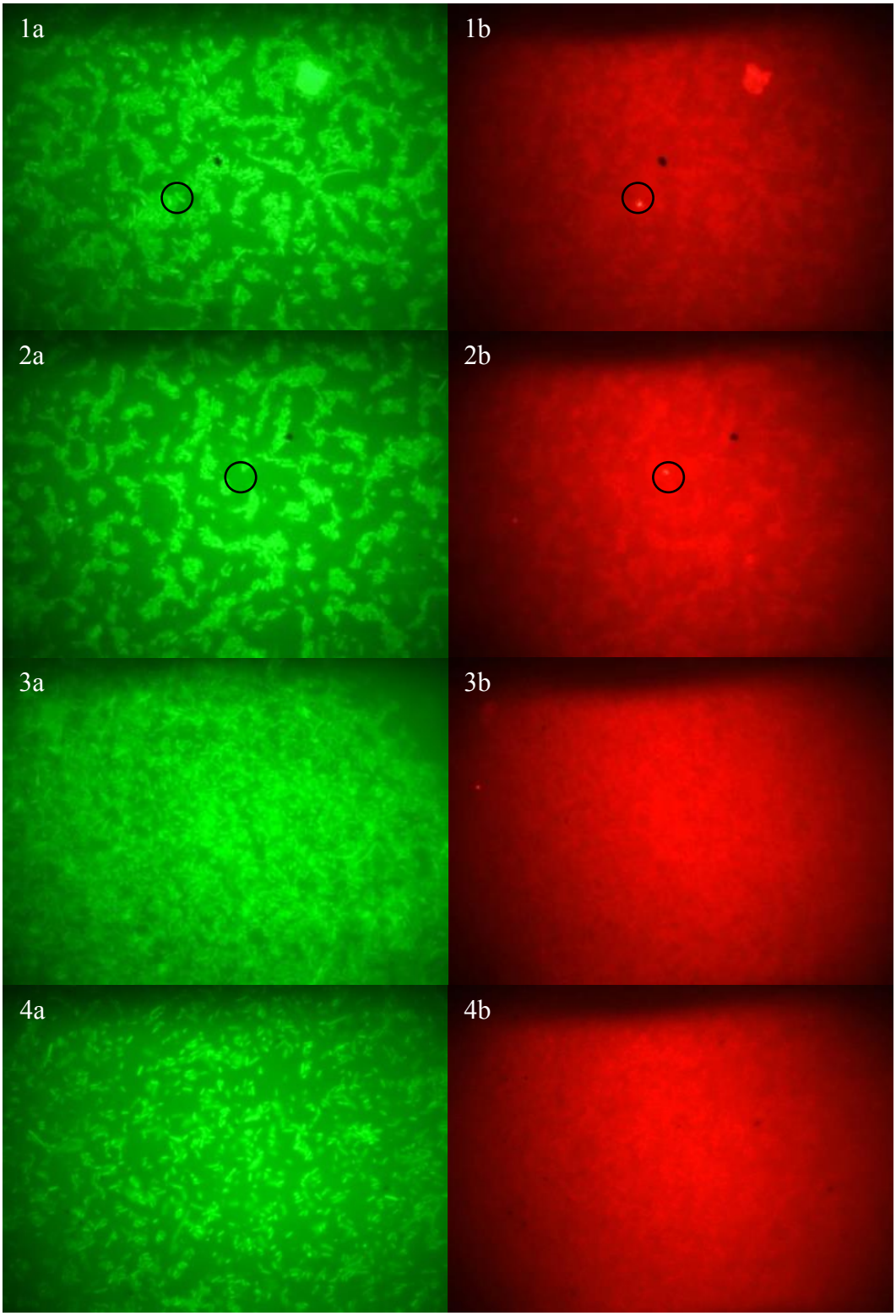

Figure 43: Texas-Red tagged Dextran (70,000) Transferred into *P. putida* UWC1 by UGT 1 & 2) test samples, transformants circled in black, 3) No Ultrasound, 4) No Dextran, a) bright field image b) WIG2 filter

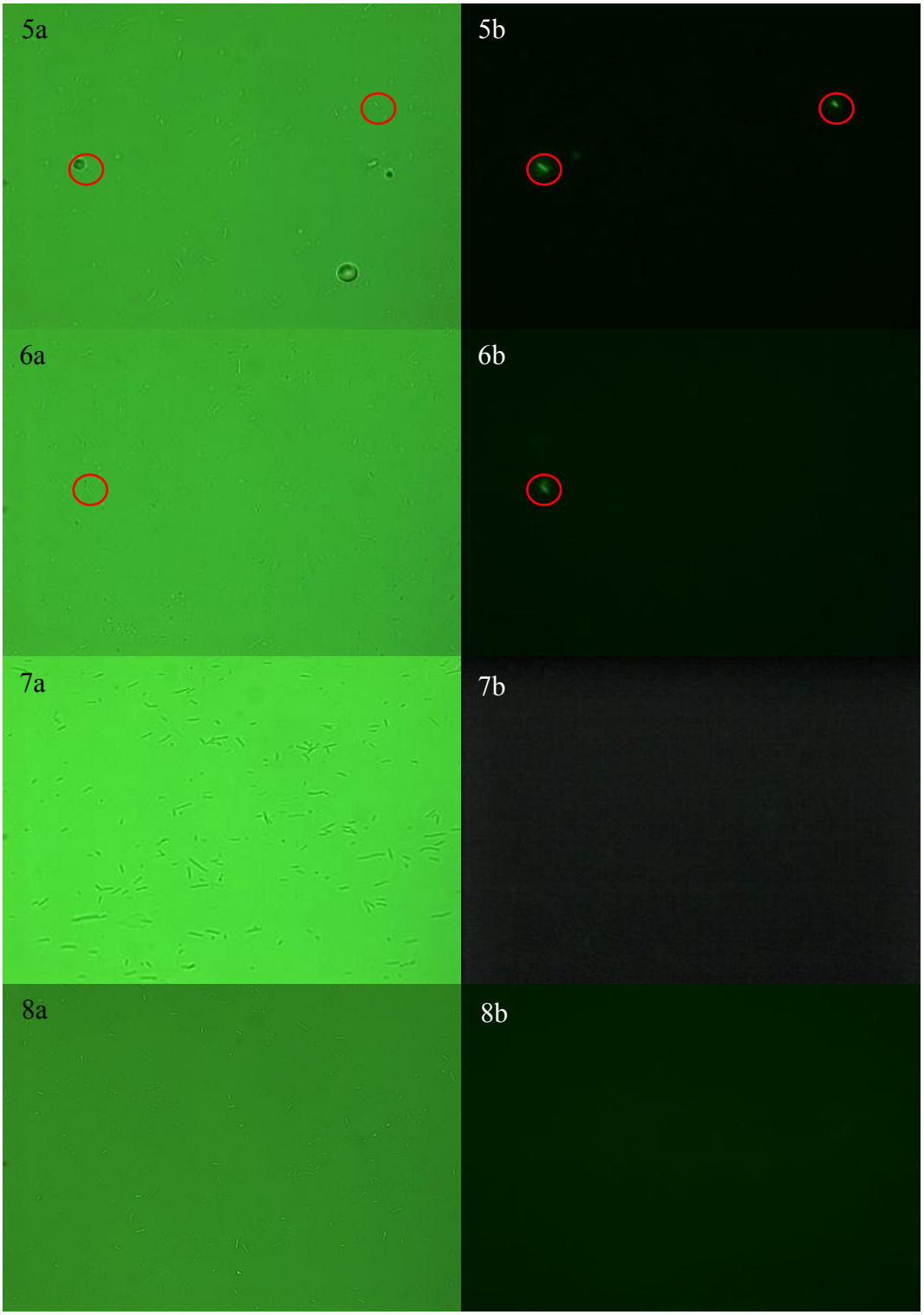

Figure 44: Fluorescein tagged Dextran (2,000,000 MW) Transferred into P. putida UWC1 by UGT 5 & 6) test samples, transformants circled in red, 7) No Ultrasound, 8) No Dextran, a) bright field image b) WIG2 filter

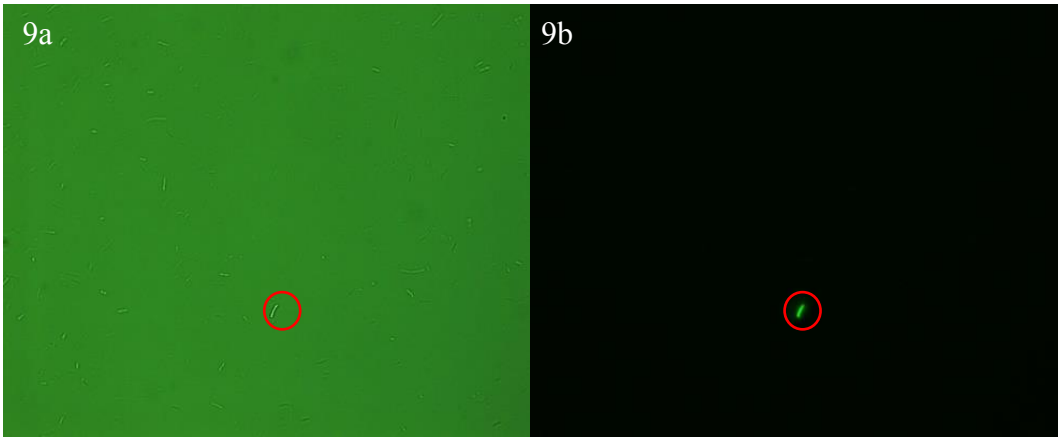

Figure 45: pBAV1K Transferred into P. putida UWC1 by UGT 9) test samples, transformants circled in red

Following statistical analysis using SPSS (IBM, USA) both dextrans are shown to be significantly different from the negative control following analysis by one-way ANOVA, (p=0.031 and p0.042 for Texas red and fluorescein respectively). No significant difference in transformant count between the two sizes of macro-molecule was detected (p>0.05).

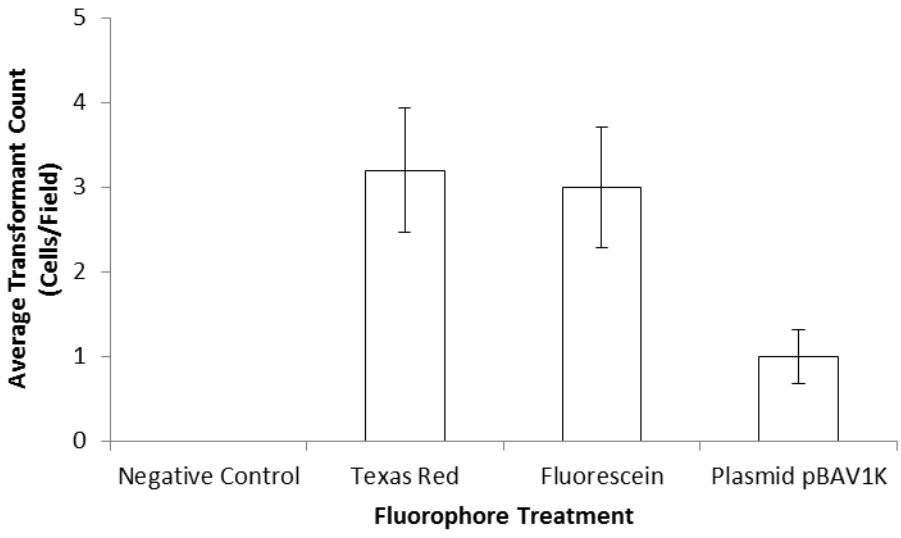

Figure 46: Transformation of Fluorophore labelled Dextran by UGT Average count per microscope field with 1 S.E. error bars

Relative cell counts give a transfer efficiency of circa 10^{-6} – 10^{-7} which is slightly higher but within the same order of magnitude as that found with plasmid transfer. Experimental limitations restrict the ability for precise enumeration of the efficiency at such small levels.

Discussion

The microscopy clearly shows fluorescent particles through the filters for both tested molecules and the positive plasmid control, which directly correspond to identifiable cells on the bright field images. This demonstrates the success of transformation of large molecules into bacteria mediated by UGT which is a novel achievement as previous work has only transferred plasmids DNA or fragments into bacteria by ultrasound.

The efficiencies were expected to be higher for the smaller molecules however at such low efficiency as is usually seen with UGT it is difficult to determine by cell counts the small variations without automation of the analysis and the computer software that was attempted to be used had difficulties distinguishing closely packed rod shaped bacteria that were found on some of the slides.

It is also notable that on examining the images there is a clear differential in bacterial morphology between those exposed to ultrasound and those without. Not only are the overall cell numbers reduced, as might be expected with the loss of a generation of doubling time due to the recovery required, but also the bacteria are generally shorter in length. It is unclear if this a long term effect on the phenotype caused by ultrasound affecting the cells and the shorter cells surviving sonication better whilst longer cells are lysed, or a shorter term effect that the community will return from once fully recovered and the shorter cells are immature and in a lag phase whilst the longer unsonicated cells are in the exponential growth phase still. This could have increased importance when investigating other species for UGT as smaller or coccoidal species maybe by more resilient to sonoporation.

Conclusions and Progression

It has been conclusively shown here for the first time that large molecule can be delivered into *P. putida* UWC1 by low frequency ultrasound with a range of molecular weight from 70,000 to 2,000,000 MW. However a differential in efficiency between sizes has not been noted as overall efficiencies are low.

This demonstrates that chemicals and drugs can be inserted into bacteria as well as plasmid DNA, creating a dramatic boost in possible applications of UGT, especially in medical and bio-manufacturing fields.

Sonicated *P. putida* UWC1 cells are noted as generally shorter than unsonicated cells however the cause and effect of this has not been investigated and further imaging of cells over a period is recommended to see how the community reacts before and after sonication along with the effect that any antibiotic selective pressure may have on morphology as this may show that cavitation has long term impacts on a community that may not all be beneficial. It is suggested that the sonication has put the cells under stress so they are not extending prior to bifurcation and once recovered will resume multiplication as normal. However the longer cells may also be more susceptible sonication damage and become lysed more easily, so naturally shorter cells or those earlier in the growth cycle may survive better and cause a shift in morphology of the community.

6: Introduction of Degradation Genes by UGT to enhance Bacterial Degradation Ability

This section demonstrates a key attribute of UGT: conferring new functions to bacteria by ultrasonically transferring a contaminant degrading plasmid into a culture of bacteria with minimal manipulation and investigating the ability of the transformants to degrade the contaminant relative to a non-transformed sample. This, along with the delivery of chemical molecules, demonstrates a practical use for UGT, both the procedure and the equipment designed and developed for this purpose.

Abstract

UGT has been employed to expand biodegradation ability by introducing a new functional gene into *P. putida* UWC1. UGT has been shown to mediate transfer of a salicylate hydroxylase gene (*sal*A), which had been inserted into a host plasmid (pBBR1MCS-2) and transferred into *P. putida* UWC1. This has proven that this method of low-frequency ultrasonic gene transfer can be successfully used to confer a new functional gene into bacteria which enables them to degrade a new contaminant with minimal manipulation of the sample. In this study UGT has enabled UWC1 transformants to degrade salicylate, which the wild type UWC1 cannot utilise. This will pave the way for the potential introduction of a wide range of degradation genes and developments towards use on environmental samples.

Introduction

The main tenets behind this development are: that biodegradation is hampered by the lack of key functional genes in the major species in an ecosystem, these genes can usually be found in the community but in different organisms, these organisms may be unculturable so the genes have not been yet been exploited (Wang et al., 2012), UGT provides a novel method than does not require laboratory culture and hence can be used for *in situ* delivery of genes into complex microbial communities. It is also feasible to use UGT at a large engineering scale whilst there is minimal disturbance to the natural surrounding community.

The introduction of salicylate hydroxylase gene (*sal*A) into *P. putida* UWC1 has been selected as a proof of concept that a degrading gene can be transferred by ultrasound and be expressed by the cell which provides transformants a selective

advantage over the non-transformed bacteria. The salicylate degrading gene (a 1.8 kb DNA fragment denoted as *sal*A in *Acinetobacter* sp. strain ADP1 (Jones et al., 2000)) encodes the production of salicylate hydroxylase which transforms salicylate to catechol, this acts as a central intermediary metabolite for continued degradation by existing aromatic cleavage genes in *P. putida* (Fuchs et al., 2011).

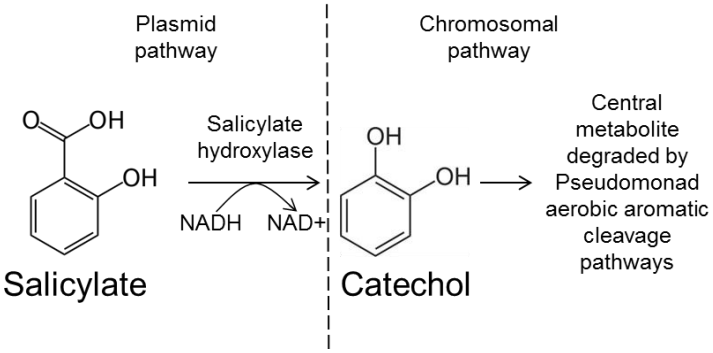

Figure 47: Degradation Pathway of Salicylate to Central Metabolite Catechol by Salicylate Hydroxylase

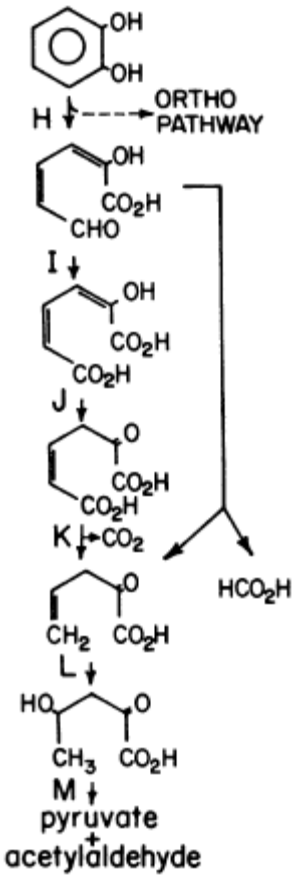

Figure 48: Oxidation of Catechol by *P. putida* UWC1 adapted from: **(Yen and Gunsalus, 1982)**

To examine the function of the introduced *sal*A in the new host, *P. putida* UWC1, the salicylate biosensor ADPWH_lux was employed to monitor the salicylate degradation process (Wang et al., 2014, Zhang et al., 2013, Zhang et al., 2012, Huang et al., 2005). Salicylate biosensor ADPWH_lux is able to quantitatively detect salicylate with a sensitivity that is comparable to GC/MS or HPLC (Defraia et al., 2008).

The advantages of using a biosensor over GC/MS or other methods is the speed (< 1hour for initial values), high sensitivity (~ nM concentrations), low cost (< 20% of EPA standard rates), simple to apply and requiring only bench-top or portable equipment, they also detect the bioavailable component making biosensors ideal for field based research and for environmental samples where the effect of the contaminants is of utmost importance. Hence ADPWH_lux is used to quantify salicylate concentrations in this study.

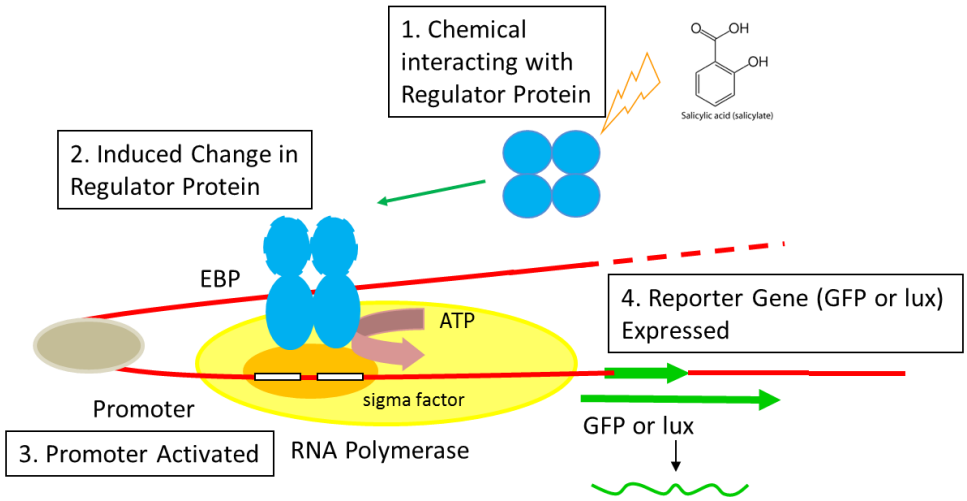

Figure 49: Mechanism of Biosensor Expression adapted from: 'Biosensor Introduction' (Huang, 2013)

The ADPWH_lux biosensor works by transcriptional regulation as shown in Figure 49. The target molecule (salicylate) diffuses into the cell and interacts with the regulatory protein, SalR (Jones et al., 2000). The specific interaction between salicylate and SalR induces a configuration change of SalR that in turn activates a promoter, P_{salA} which is fused with a bioluminescence gene cluster, *lux*CDABE. In the presence of salicylate ADPWH_lux is induced to express this bioluminescence gene cluster, the intensity of bioluminescence is proportional to the concentration of

salicylate (Huang et al., 2005). Thus by use of the biosensor at a series of time points the change in salicylate concentration can be determined.

Materials and Method

The 1.8 kb *sal*A fragment coding for the salicylate hydroxylase was amplified from *Acinetobacter* sp. Strain ADP1, PCR was performed on a colony picked from overnight agar plate growth with primers "salA_EB_for and salA_EB_rev" both containing EcoRI and BamH1 restriction sites (see Figure 50). PCR reaction mixture used: 38.5 µl dH2O, 5 µl PCR buffer x 10 (Fermentas), 2 µl 5 mM dNTPs, 2 µl 5 µM salA_EB_for, 2 µl 5 µM salA_EB_rev, 0.2 µl ADP1 and 0.3 µl Taq polymerase (Sigma).

salA_EB_for:

GATGCTATTTTAGGGAGAATTCCACGGATCCAGTGTAAGT (EcoRI, BamH1)

salA_EB_rev:

ACTTACACTGGATCCGTGGAATTCTCCCTAAAATAGCATC (EcoRI, BamH1)

Figure 50: *sal*A Primer Sequences

PCR was purified using a QIAquick PCR Purification Kit (QIAGEN, UK) following the manufacturer recommended procedure. The PCR purification products were digested in parallel with the recipient plasmid, pBBR1MCS-2, with restriction endonucleases EcoRI and BamH1 for 2 hours at 37 °C to produce fragments with compatible sticky ends. The digested products were separated by agarose gel electrophoresis and cleaned using a QIAquick Gel Extraction Kit (QIAGEN, UK) following the manufacturer recommended procedure. Having produced suitable digested fragments these were ligated using T4 DNA ligase (NEB, UK), once incubated the plasmid formed (henceforth referred to as pBBR1_salA) was transferred to *E. coli* DH5α by heat-shock. The resultant cells were grown on LB agar supplemented with 50 µg/ml kanamycin sulphate and 1 mM salicylate to isolate transformants. Transformants that were able to grow on LB kanamycin and produced brown colouration (catechol from salicylate degradation) were picked and incubated in LB broth with Km_{50} and the plasmids extracted by Miniprep (QIAGEN, UK).

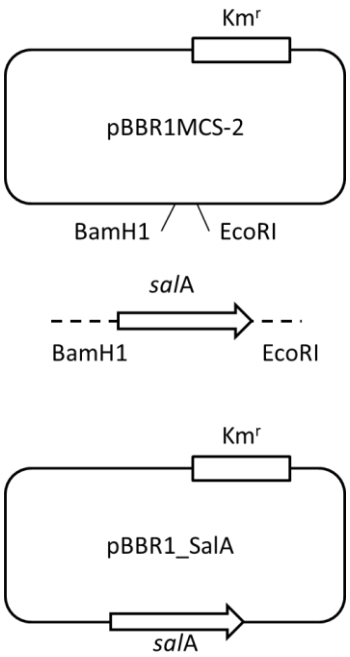

Figure 51: Insertion of *sal*A into pBBR1MCS-2

The extracted plasmid, pBBR1_salA was then used for UGT, this was performed using the direct immersion procedure; using 20 ml of *P. putida* UWC1 from overnight growth with 100 µl of extracted plasmid (pBBR1_salA), 5 replicates were performed with positive controls using pBBR1MCS-2 and negative controls with either no ultrasound or no plasmid. Samples were sonicated for 3 minutes at 27.5 kHz, followed by 2 hours recovery at 27 °C. 100 µl of each sample was spread on LB agar plates supplemented with 50 µg/ml kanamycin and 1 mM salicylate, a further 100 µl was spread on minimal media plates with 2 mM salicylate as the sole carbon source and a 10^{-8} dilution was spread on LB agar, all samples were incubated overnight at 27 °C at colonies counted the following day.

To investigate the growth curve and effects of the added degrading genes transformants and controls were also inoculated into fresh LB broth supplemented with 2 mM salicylate (with 50 µg/ml kanamycin for both pBBR1MCS-2 and pBBR1_salA containing strains) and the growth was recorded over a 24 hour period by measuring OD600 using a clear 96 well micro-plate and read with a Synergy II Multi-mode Microplate Reader (BioTek, USA).

To assess the degradation of the salicylate by the transformed cells *Acinetobacter* ADPWH_lux whole cell biosensor was used. Biosensor stock solution of

Acinetobacter in sodium chloride solution (0.85%) was pre-prepared ready for use and stored at 4 °C for use and *P. putida* UWC1 transformants containing pBBR1_salA were incubated overnight in LB broth with 2 mM salicylate and 50 µg/ml kanamycin to maintain selective pressure as were negative controls of *P. putida* UWC1 with pBBR1 and plasmid-free *P. putida* UWC1 (without Km). Cells were washed thrice by centrifugation and re-suspension in MM to remove any trace of kanamycin as this was found to interfere with the non-resistant *Acinetobacter* strain in preliminary experimental testing.

Stock salicylate standards were prepared to generate a calibration curve for the biosensor with salicylate concentrations of 0, 10, 20, 50, 100 and 200 µM dissolved in sterile UHQ water.

The test samples and controls were then prepared to a final volume of 20 ml, comprising: 2 ml LB broth (i.e. $1/10^{th}$ concentration), 1 ml washed overnight growth of sample, 40 µl 1 M salicylate (final concentration 2 mM) and ~17 ml minimal media. Once prepared time zero samples were taken and the remainder incubated at 27 °C between the sampling points at 0, 2, 4, 12, 16, 18, 20 and 24 hours, further samples and readings were then taken at 58 hours and finally after 14 days. Each time point sample was diluted 1:20 to be within the mid-range of the biosensor for optimum accuracy.

For operation of the biosensor: the stock solution is pelleted and re-suspended in minimal media with 1 M sodium succinate and incubated for 10 min at 30 °C. 20 µl of the biosensor suspension is then added to each well of a 96 well black micro-plate with a clear bottom (Corning, USA) (n.b. black is used as opposed to the usual white plate for luminescence due to the reduction in interference between wells with the black plates). 180 µl of each diluted sample is then added: 3 replicates for each bacterial sample (of which there are 3 replicates of *P. putida*, *P. putida* with pBBR1MCS-2 and *P. putida* with pBBR1_salA grown from individual agar colonies) and 3 replicates for each calibration standard of salicylate. The micro-plate was then inserted into the reader (Synergy II, BioTek, USA) and programmed to read luminescence and OD_{600} every 5 minutes for 2 hours with 30 seconds shaking prior to each reading. Data was then extracted to Excel (Microsoft Corp. USA) for analysis.

Results

The *Sal*A fragment was successfully cut and ligated into plasmid pBBR1MCS-2 to generate the plasmid referred to as pBBR1_salA.

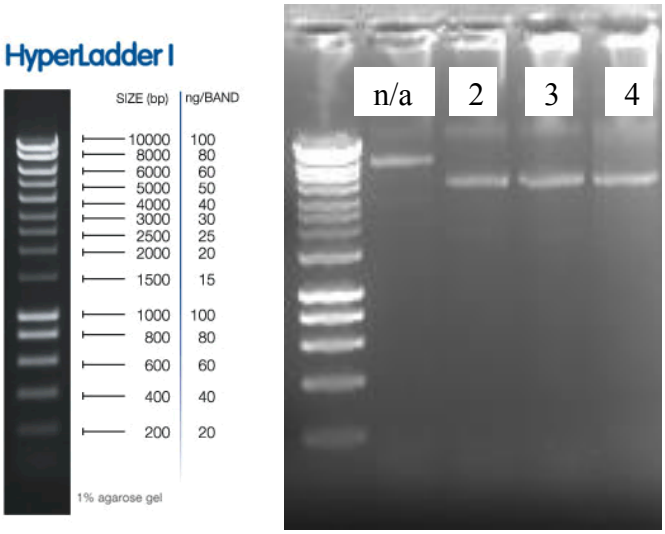

Figure 52: Agarose Gel Image of pBBR1_salA (lanes 2-4)

This plasmid fragment was in turn transferred into *E. coli* DH5α by heat-shock and selected for with kanamycin. Transformants from the heat-shock and controls of pBBR1MCS-2 (taken from -80 °C) stocks both have grown on agar plates supplemented with salicylate (1 mM) and kanamycin (50 µg/ml) for selection. However pBBR1_SalA is able to also degrade the salicylate producing catechol, hence the brown colouration on plate 2 (see Figure 53).

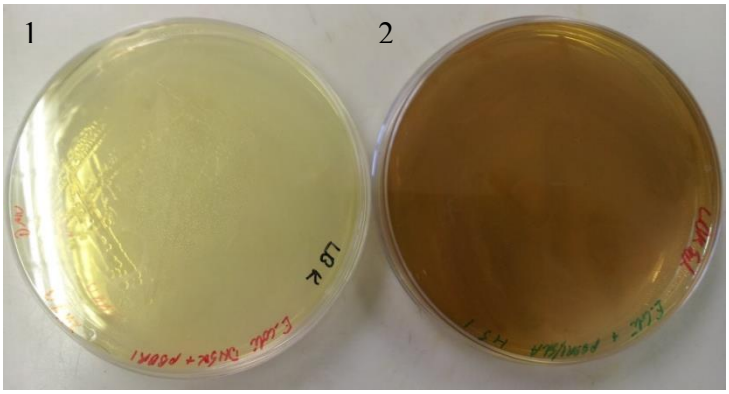

Figure 53: Plasmids (pBBR1MCS-2 and pBBR1_salA) inserted into E. coli by heat-shock grown on LB agar plates with 50 Mg/ml kanamycin and 1 mM salicylate 1) pBBR1MCS-2 inserted, 2) pBBR1_salA inserted (pBBR1MCS-2 does not degrade salicylate, pBBR1_salA degrades SA to catechol – brown colour produced)

The extracted plasmid, pBBR1_salA was transferred by UGT into *P. putida* UWC1 producing transformants (small white colonies) successfully growing on minimal media agar plates with salicylate as the sole carbon source (see Figures 54 and 55) and none of the negative controls produced any colonies. When grown on LB agar with kanamycin selection both the transformed pBBR1_salA samples and the pBBR1MCS-2 controls grew and the plasmid free or unsonicated ones did not. The transfer rates were not dissimilar with the larger pBBR1_salA plasmid just under an order of magnitude less efficiently transferred (see Table 6 for transfer efficiencies).

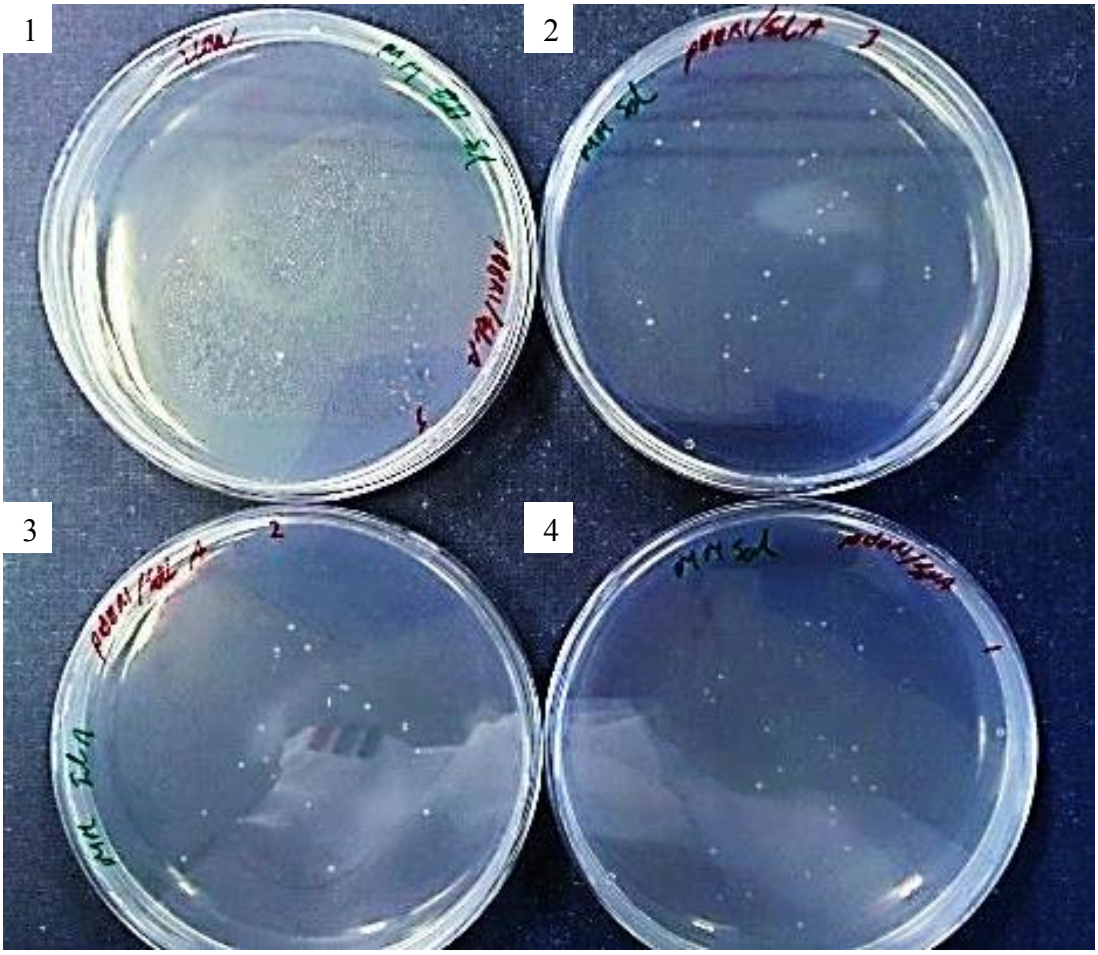

Figure 54: *P. putida* UWC1 with plasmid pBBR1_salA inserted by UGT MM agar plates with 50 µg/ml kanamycin and 1 mM salicylate as the sole carbon source, 1-5) pBBR1_salA, 6) pBBR1MCS-2 7) No plasmid 8) pBBR1_salA without sonication (see also **Figure 55**)

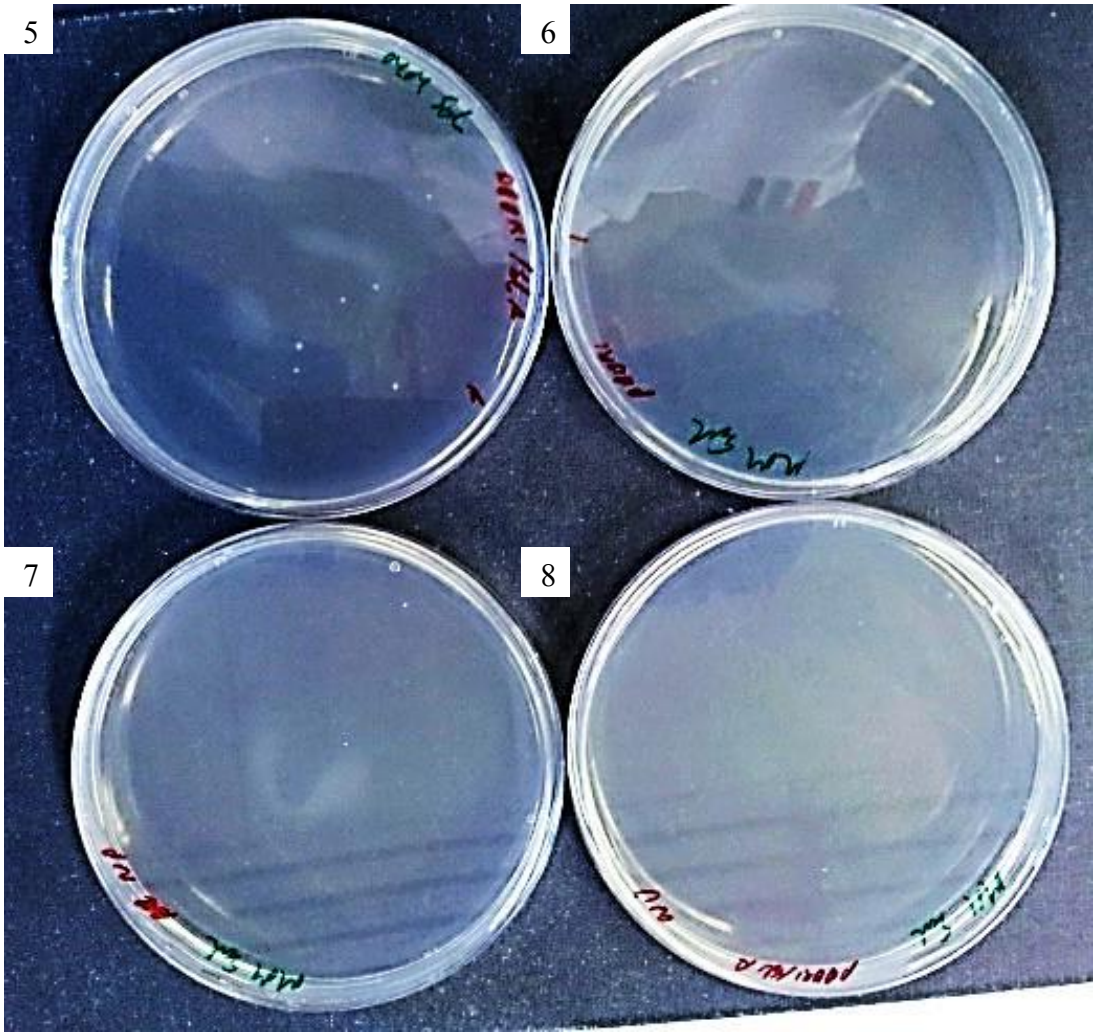

Figure 55: *P. putida* UWC1 with plasmid pBBR1_salA inserted by UGT MM agar plates with 50 µg/ml kanamycin and 1 mM salicylate as the sole carbon source, 1-5) pBBR1_salA, 6) pBBR1MCS-2 7) No plasmid 8) pBBR1_salA without sonication (see also **Figure 54**)

Table 6: Transfer Efficiency of pBBR1_salA into *P. putida* UWC1 by UGT average of 5 replicates with 1 S.E.

Plasmid Transferred	Transformation Efficiency	
	MM agar with 2 mM salicylate	LB agar with 50 mM kanamycin and 2 mM salicylate
pBBR1_salA	$5.44 \pm 4.99 \times 10^{-9}$	$8.31 \pm 6.50 \times 10^{-9}$
pBBR1MCS-2	0	$5.5 \pm 0.856 \times 10^{-8}$
No Plasmid	0	0
pBBR1_salA without sonication	0	0

Following showing the transfer efficiency rates of degradation were investigated using cells grown in LB broth with a salicylate supplement. The initial growths curves are similar however the wells containing the pBBR1_salA plasmid were able to continue to survive after the LB broth had been exhausted, shown by the continued stationary phase after 18-20 hours (see Figure 56) for the test sample whilst the control samples began to deteriorate.

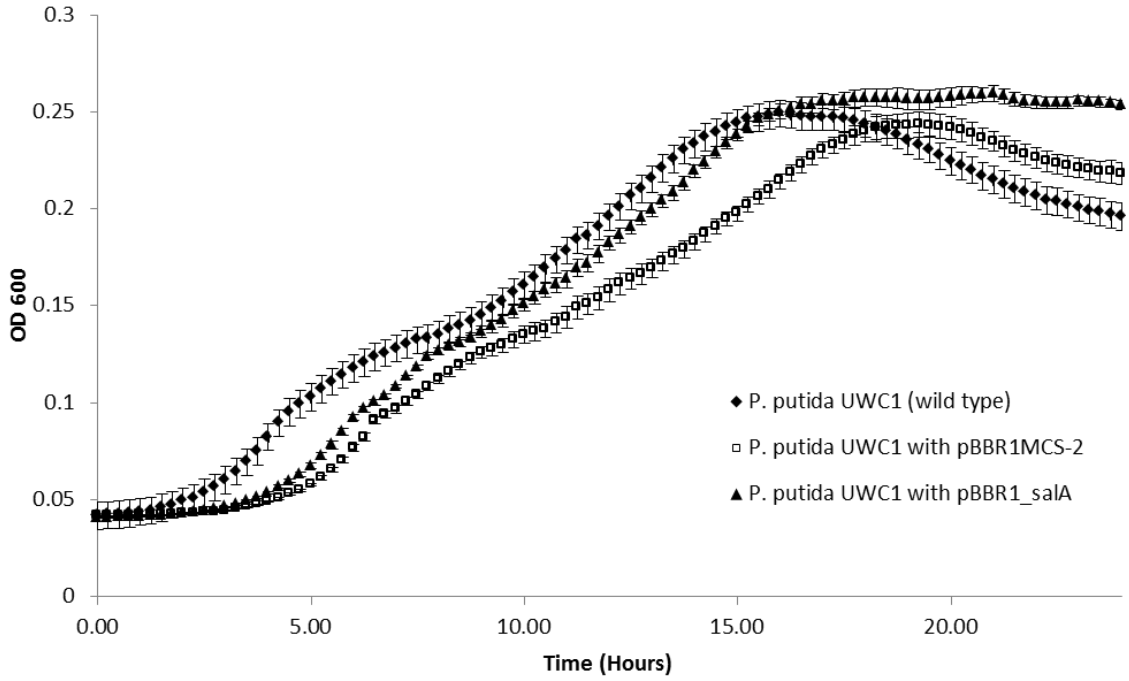

Figure 56: Growth rate of *P. putida* UWC1 with pBBR1_salA inserted by UGT (▲) compared to pBBR1MCS-2 (□) and no plasmid (♦) grown in 1/10 concentration LB broth supplemented with 2 mM salicylate and 50 µg/ml kanamycin for plasmid selection, average of 10 replicates ± 1 S.E.

To then determine the degradation rate the biosensor was used to quantify the concentration of salicylate at the selected time points. The biosensor is sampled over a 2 hour period to allow the bioreporter cells to adjust to the concentration of salicylate available and reach peak luminescence, usually after approximately an hour but for higher concentrations there is a longer delay to reach maximum luminescence. The resultant maximum luminescence from the data points for each sample was collected and plotted to give a calibration curve for each time point (see Figure 57) showing the differential in luminescence for the salicylate dose. A calibration curve is required for each time point to prevent inconsistencies between

batches of biosensor. To calculate the salicylate concentration in each sample the value is interpolated from the data using the maximum measured luminescence:

$$S_x = \frac{(L_x - L_1)}{\left(\frac{L_2 - L_1}{S_2 - S_1}\right)} + S_1$$

Equation 9: Interpolating salicylate concentration from luminescence

Where:

S_x = unknown sample salicylate concentration

L_x = maximum sample luminescence

L_1 = lower bound of luminescence

L_2 = upper bound of luminescence

S_1 = lower bound of salicylate concentration

S2 = upper bound of salicylate concentration

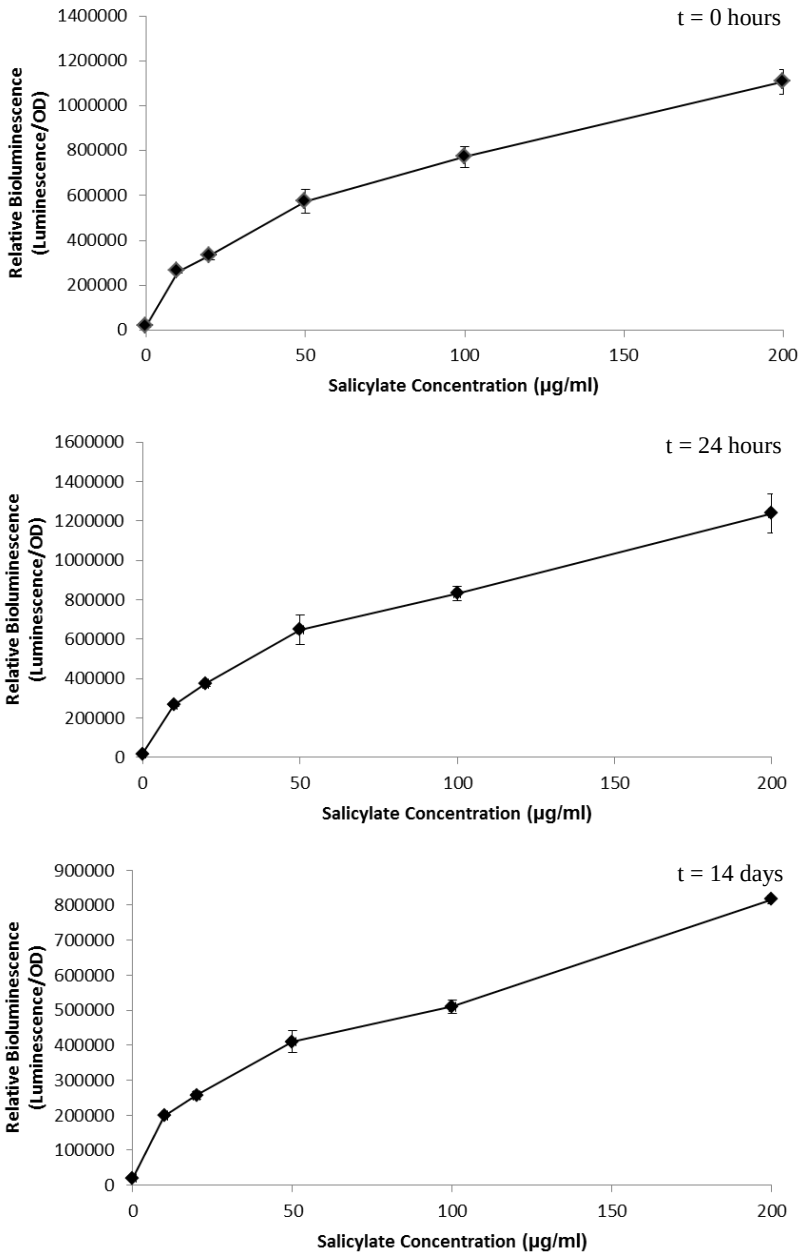

Figure 57: Salicylate Biosensor Luminescence Calibration Curves for 0 hours, 24 hours and 14 days, average values of 3 replicates with 1 S.D. error bars

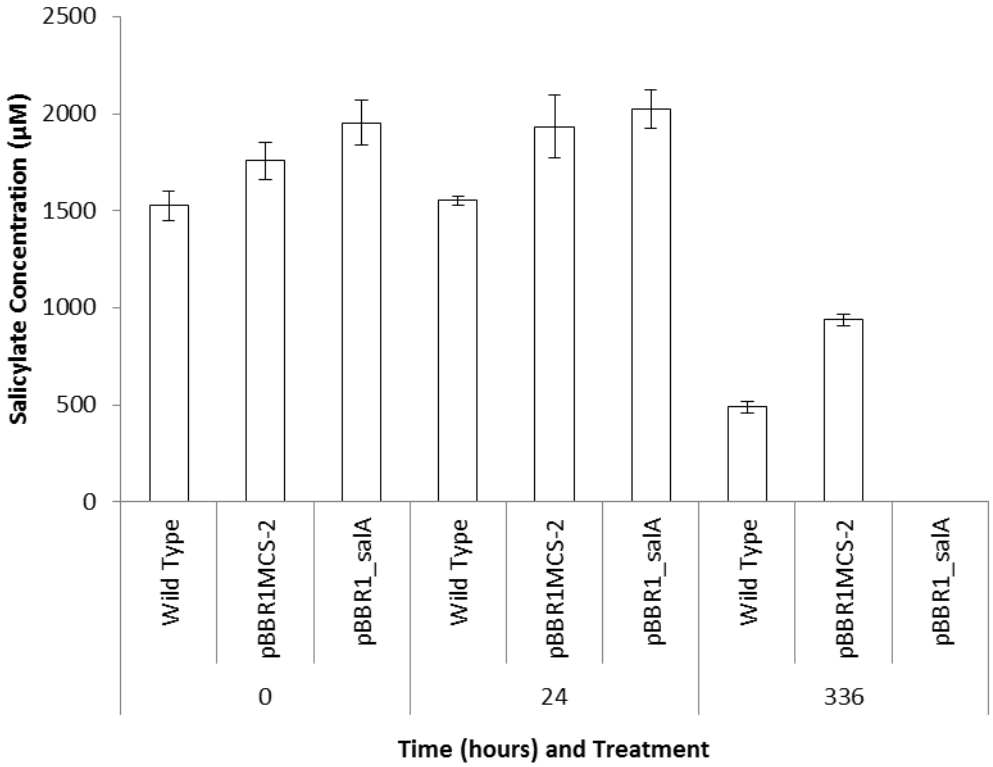

Figure 58: Degradation of Salicylate by *P. putida* UWC1 with pBBR1_salA inserted by UGT compared to pBBR1MCS-2 and wild type with no plasmid at 0, 24 and 336 hours

Initially no degradation was observed up to 24 hours so the samples were left for a longer period prior to the final sample at 14 days, at which point a colour differential between the samples was observed (see Figure 59). At 14 days it can be seen that the sample with the plasmid, pBBR1_salA, the 2 mM of salicylate is fully degraded (< 1 µM) whilst without the plasmid or with the unmodified pBBR1MCS-2 plasmid only partial degradation has occurred (490 & 930 µM remaining respectively).

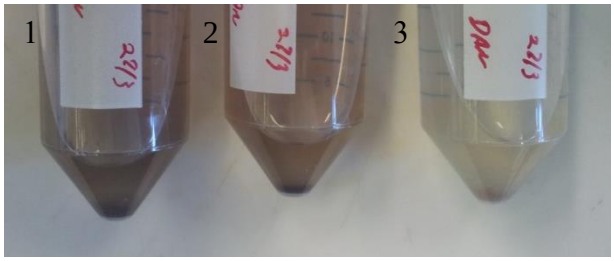

Figure 59: Colouration Differential of Salicylate Degradation after 14 days by *P. putida* UWC1 containing: 1) No Plasmid, 2) pBBR1MCS-2, 3) pBBR1_salA, image showing 1 of 3 replicates for each sample

Discussion

Here it is seen that *P. putida* UWC1 is unable to use salicylate as a sole carbon source due to the lack of the key functional gene to convert salicylate to catechol, however it is then naturally able to degrade catechol to pyruvate (Yen and Gunsalus, 1982) so by introducing this initial degradation step UWC1 can then degrade salicylate.

The successful fragment isolation, PCR, cutting and ligation of the *sal*A gene into pBBR1MCS-2 to produce pBBR1_salA and the resultant transfer into *E. coli* by heat-shock provided the required genetic material for the attempted ultrasonic transfer into *P. putida* UWC1. The heat-shock products where easily identifiable following selection with kanamycin and the use of salicylate to confirm, as the *sal*A fragment meant that the transformed colonies produced a brown colouration of catechol which the *E. coli* was unable to further degrade; this ensured no self-ligated pBBR1MCS-2 plasmids with kanamycin resistance had been transferred.

The UGT itself also ran smoothly and whilst the efficiency was lower for the *sal*A containing plasmid, this is not unexpected as it is 1.8 kb larger than the original pBBR1MCS-2 plasmid. The transformants were able to satisfactorily grow with salicylate as a sole carbon source, albeit at a slower rate to that found when grown on LB due to the nutrient differential and selective pressure ensured the plasmid was retained. No colouration change was seen with the minimal media as the *Pseudomonas* strain was able to degrade catechol, resulting in the formation of a full degradation pathway for salicylate that was previously not available in this strain.

To assess this novel degradation capability for this strain the OD600 analysis showed across all the replicates that after the dilute LB broth in the sample had been consumed (approx. 16-18 hours) the *sal*A containing samples were able to maintain themselves in the stationary phase for at least a further 4 hours before the 24 hour analysis was completed. Longer analyses were not possible as after this time the samples in the microplate wells began to dry out and changes to the set-up would affect the data quality.

It can also be seen that the plasmid free sample had a shorter lag-phase, relative to the plasmid bearing bacteria, prior to exponential growth, most likely due to the lack of antibiotic requiring the increase in energy consumption to replicate and express the resistance plasmid. However the pBBR1_salA samples catch up in growth rate suggesting that they are already making use of the added carbon source despite the presence of the preferred LB nutrients. This could be due by the plasmid already being expressed for the kanamycin resistance and hence inducing the Salicylate degradation simultaneously. It is clear that the salicylate is not a preferable carbon source for growth as it is only able to maintain the OD600 without further growth.

The inference from this parallel metabolism is that when used in an environmental sample with a suitable inducer the bacteria will be able to express the plasmid and degrade the contaminant even with other available carbon sources, thus conferring an eventual advantage to the transformed cells.

Growth experiments for both OD600 and degradation using minimal media and salicylate as a sole carbon source where found to be slow and exceeded the useful parameters of the equipment used to test and analyse the samples, however the 1/10 dilute LB provides a comparable equivalent to generate robust data to prove the effectiveness of the transformation.

Having shown that the ultrasonically transformed cells were able to grow using the salicylate as a carbon source, the work developed to investigating the degradation aspect. Bioreporter cells were used to monitor the rate of degradation.

The time 0 hours biosensor results were as expected giving an approximate concentration of 100 µm which allowing for the dilution factor matches the 2 mM of salicylate at the start, there is some aberration from exactly 100 µM that with further

familiarity with the biosensor preparation and operation is expected to be reduced. The 24 hour time point shows a slight increase in the salicylate concentrations which is theoretically impossible however suggests that there is bioaccumulation of contaminant within the cells as the nutrients from the dilute LB media in the sample have been exhausted and the bacteria become increasingly dependent on the alternative carbon source or that the biosensor is also reacting to some of the metabolites produced during the salicylate degradation (e.g. catechol).

After 14 days it can be categorically shown that all the salicylate in the pBBR1_salA samples has been degraded with none of the samples showing quantifiable levels of the contaminant over a 1 µM threshold, whilst a reasonable proportion remains in the control samples. However it is unexpected to see such levels of degradation in the control samples showing some partial degradation of the contaminant, the cause of this is unknown, whether due to an alternate biological pathway naturally occurring within the *Pseudomonas* strain that is not sufficient to sustain growth but allows break down of the salicylate as a co-metabolite, or whether as the bacteria in the incubated large sample die they accumulate the salicylate within the cells reducing the bioavailability of the contaminant when the sub-sampling is performed for use with the biosensor and therefore the biosensor is unable to react to this proportion. Or whether there is a non-biological pathway due to light degradation or other complexes being produced in the sample reducing the bioavailability of the salicylate. This shows the combined strength and weakness of the ability of the biosensor to only detect bioavailable compounds against GC/MS, however if the contaminant is not bioavailable then the risk to the environment is markedly reduced.

To further assess the effectiveness of transfer it is suggested that parallel testing with other degrading species is performed to investigate the degradation rate following UGT, which would further show any advantages of UGT over existing bioremediation treatments.

Conclusions and Progression

Having successfully designed primers, isolated and extracted the *sal*A gene for salicylate degradation from *Acinetobacter* sp. strain ADP1, then inserted it into plasmid pBBR1MCS-2, which has regularly been used in the UGT work until this point, this plasmid dubbed ‘pBBR1_salA’ was replicated for use in UGT. The

plasmid was then successfully transferred into *P. putida* UWC1 by direct immersion UGT as shown by growth on minimal media with salicylate as a sole carbon source. Further investigation using whole-cell biosensor technology has shown that the transformed cells can completely degrade salicylate in the sample whilst after two weeks a large proportion remains in the non-transformed cells. Hence UGT has conferred a novel degradation pathway to *P. putida* UWC1 enabling it to degrade a contaminant that it was previously incapable.

The whole cell biosensor technology has allowed for high quality data to be quickly and cheaply produced and complemented the aims of this work to develop a simple method to improve biodegradation; opening the door for a cohesive method of assessing, treating and monitoring contamination using biological techniques and avoiding the high overheads of technical apparatus and chemicals.

This successful UGT and expression of degradation genes into *P. putida* UWC1 demonstrates that the process can transfer more than marker genes, and be inserting suitable degrading gene fragments any contaminant can potentially be bioremediated by this technique. UGT is still currently restricted to a few species and *in situ* or environmental samples still require further development and this is the key to unlocking the technology beyond a successful micro-scale laboratory technique.

7: Conclusions

This thesis has achieved a series of major goals and provided a progression from the pioneering initial experiments towards the ultimate target of *in situ* gene transfer for bioremediation of contaminated land. The work has progressed from investigating the optimum transfer frequency to design and manufacture of a unique variable frequency sonotrode apparatus and for the first time proving that cavitation is the mechanism driving UGT.

This apparatus has then successfully transferred a new functional gene (salicylate hydroxylase) into *P. putida* UWC1. This has proven that this method of low-frequency ultrasonic gene transfer can be successfully used to confer a new functional gene into bacteria which enables them to degrade a new contaminant with minimal manipulation of the sample at industrial and engineering scale.

The key achievements are:

1. Design, development and manufacture of the variable frequency sonotrode apparatus
2. Optimization of the procedure with respect to frequency
3. Identification that cavitation generated by the collapse of micro-bubbles is the mechanism behind UGT
4. Scaling the technique from a microlitre volume to a millilitre volume, potential for further increase and the optimization of exposure time for a larger volume
5. Delivery of a range of marker plasmids into *P. putida* UWC1
6. Delivery of macro-molecules into *P. putida* UWC1
7. Delivery of contaminant degrading genes into *P. putida* UWC1, demonstrating the gene expression and ability to degrade a contaminant that the bacteria was previously unable.

Other findings from this research also include:

1. Plasmid concentration is the major limiting factor for UGT in high nutrient conditions. Consequently the highest practical plasmid concentration is recommended.

2. UGT is markedly reduced under low nutrient conditions under the experimental setup used. Dosing with SOC or other high nutrient media following UGT may aid recovery and gene expression.
3. There is a wide variation between species as to their predisposition to transformation by UGT under the conditions used. Varying the exposure parameters may be needed for successful transfer to other species.

The development of the apparatus has produced an effective sonotrode that enables direct immersion of the tip of the sonotrode into the sample thus removing the necessity for specific vessels or apparatus in which the sample must be prepared. This has allowed the procedure to be scaled from the microlitre scale, comparable to other laboratory techniques, to the millilitre scale with scope for further increase reaching an industrial engineering scale. This apparatus and larger scale of sample has then been optimised for ultrasound frequency and exposure time, and the effect of plasmid concentration, cell OD600 and cell growth phase was investigated, with the plasmid concentration being shown to be a key limiting factor; however increases in plasmid volumes are costly to prepare. Techniques to maintain high concentration of plasmids around cells with lower cost were reviewed, for example: chemical treatment or modification of the plasmids to enhance attachment to cells or encapsulation of a single cell and a few plasmids in a micro-compartment or micro-fluidic device to ensure a high ratio of plasmids to cells in millions of closed mini-systems (e.g. an emulsion) and investigation is recommended for future work

The stationary phase was identified to be the preferred growth phase for UGT which makes UGT ideal for environmental application as the indigenous species targeted will generally be in an equilibrium state. Throughout experimentation it is important to note that 50 mM $CaCl_2$ concentration is used and this ionic strength is shown to be key in aiding a higher efficiency of UGT, whether this can be transferred to large scale environmental samples will depend on the existing conditions and may require a sample of the ecosystem to be isolated, treated with $CaCl_2$, sonicated then re-introduced.

This study shows, for the first time, that DNA/RNA/macro-molecules can be delivered into bacterial cells by micro-bubble induced cavitation. The identification and proof of micro-bubble induced cavitation as the mechanism behind UGT was

noted first when only a few frequencies produced high rates of transfer and these matched the frequencies were agitation occurred in the samples. This observed agitation was seen again on scaling the procedure and a micro-bubble cloud could be seen, following testing either side of this frequencies it was concluded that this was the driver behind UGT. Literature analysis of micro-bubbles and their collapse led to the hypothesis that the mechanism was that microjets of high pressure, generated when the cavitation micro-bubbles collapse, create pores in the cell walls enabling the plasmid DNA to cross the boundary into the cell, and proving the damage is not too great they are able to repair the pores having taken up the plasmid, which is then expressed by the bacteria.

This successful delivery and understanding of the mechanism was then applied to the delivery of not only DNA but also macro-molecules into the bacteria, two fluorescent tagged large dextran molecules (70,000 MW and 2,000,000 MW) were successfully transfer by ultrasound into *P. putida* UWC1, demonstrating the capability of UGT to deliver macro-molecules into bacteria which could be used for delivery of drugs or high molecular weight chemicals. This can potentially enable delivery of bioparts and nanomaterials for synthetic biology to targeted locations in an organism.

Delivery of marker genes into environmental samples has not been successfully demonstrated as despite some optimistic results introducing a GFP marker plasmid into a biosludge sample from a coke oven effluent treatment plant, no transformants could be isolated to confirm the gene had been introduced. Similarly for the attempted transfer of an mCherry marker plasmid into a concentrated river water sample no transformants could be identified.

One of the main benefits of performing an *in situ* transfer is that there will already be a selective pressure present in the environment for the gene and the added capability to degrade a contaminant that the bacteria was previously unable will give the transformed bacteria a competitive advantage. This was demonstrated using antibiotic resistance where following UGT of a resistance plasmid into *P. putida* UWC1 the transformants became dominant within 7 days with a strong selective pressure and were able to maintain a stable population with even only a weak selective pressure.

Lastly UGT successfully mediated the introduction of a functional gene into a bacterial strain enabling the degradation of a contaminant that the strain was previously unable to use. A salicylate hydroxylase gene (*sal*A) was transferred by ultrasound into *P. putida* UWC1, this enabled the UWC1 to utilise salicylate as a sole carbon source for growth whilst untransformed cells were unable to survive, as well as giving an advantage to the transformed cells over the wild type, enabling them to grow faster than cells without the gene and survive for longer when grown in a mixed carbon source environment.

The project has taken many successful steps towards *in situ* application of UGT for environmental bioremediation. Whilst the final goal has yet to be attained and the technique is still a way from commercialisation it remains a technology with a bright future and high likelihood of realization. To make these final steps future progression is recommended to focus on environmental samples using selective pressure to encourage even the lowest transfer rate to become dominant and identifiable. It is recommended to introduce degradation genes into the environmental samples and to monitor degradation rates as this was unfortunately not attempted within the scope of the thesis. When using environmental samples the ionic concentration is expected to be a key factor in transfer as the $CaCl_2$ concentration was found to be important using LB media and hence a different host environment is expected to need a variation in this.

To further the understanding of the exact mechanism of transfer it would be useful to examine the formation and collapse of the cavitation microbubbles using a high speed magnifying camera, and then investigate the interaction between the collapse and the bacterial cell walls and how they are damaged or affected to allow UGT to occur, whilst being isolated from the vibrations produced by the apparatus.

8: Recommendations and Future Work

The results of this thesis confirm that the technology developed has enormous potential and further developments in the both optimising the procedure and exploring the scope of applications of the technology will be required to realize this.

The key areas to target for development would be:

1. Source a suitable high-speed microscopic camera to image the cavitation and subsequent effect on the bacteria to fully understand the mechanism and sonoporation leading to UGT and then monitor the cell recovery to gain an insight into how best to apply the technique to differing environmental conditions.
2. Develop uses for macromolecule delivery, specifically for using bacteria as cargo carriers to potentially deliver drugs or chemicals *in vivo*, and being lysed (again by ultrasound) when they reach their target to release the payload.
3. Miniaturising the apparatus to a microfluidic device to allow single cells to mix with plasmids and sonicate them in a confined setup to achieve a high transformation efficiency that could easily be automated. A steady flow of cells and plasmids could be mixed and passed through a microbubble stream for exposure to the cavitation events. This could be especially useful when applied to a bioreactor ensuring the degradation capability is maintained throughout cycling of the waste being treated.
4. Conversely enlarging the apparatus for large scale delivery using an array of sonotrodes to enable delivery to larger volumes of bacterial suspension especially with a view to the environmental applications where large volumes will require treatment. This will require careful consideration of how the ultrasonic wave fronts may interact and monitoring of any adverse effects especially heating.

Areas from this work that require confirmation or further investigation to understand the parameters and factors involved between the success and failure of UGT experiments.

1. Measure the calcium content in all media used in transformation to quantify the amount of ionic Ca^{2+} present during sonication. Along with a more general assessment of the ions so their effects on UGT can be managed when varying conditions are met in environmental samples.
2. Check plasmid compatibility and investigate broad range plasmids to ensure that plasmid DNA introduced by UGT can be adequately expressed by the transformants.
3. For environmental samples consider using positive control replicates using samples dosed with *P. putida* UWC1, this should provide a confirmation that salt and recovery conditions are suitable for UGT and hence eliminate another unknown from the transfer.

After submission it is intended that the following papers are generated from this research:

1. UGT in bacteria is mediated by microbubble cavitation: this will be based upon the data found in Section 3.3, concerning the confirmation of cavitation as the driver for UGT found using the second generation sonotrode. The hypothesis is that UGT occurs solely at resonant frequencies where cavitation is present during sonication. This is clearly demonstrated in the data set and provides a key understand for the mechanism behind low frequency UGT.
2. Macromolecule delivery into *P. putida* UWC1 by ultrasound: this will use the data set found in Section 5 to examine the hypothesis that the UGT method can be used to not only transfer DNA but other macromolecules into bacteria using low frequency ultrasound. This will enable the delivery of drugs and other large chemical compounds into bacterial cells and it is demonstrated by the data that a range of molecular weight molecules can be delivered.
3. Conferring Salicylate degradation capability into *P. putida* UWC1 by UGT: based upon the research undertaken for Section 6. This will encapsulate the creation of the plasmid and its subsequent transfer and analysis of degradation of salicylate using the *Acinetobacter* biosensor to examine the hypothesis that UGT can be used to deliver novel degradation genes into bacteria to promote degradation.

These are just a few of the areas that this exciting new technology can be applied to, to potentially versatility of UGT over other transfer techniques is its major feature and providing the simplicity of the delivery can be maintained and demonstrated for a wide range of species this could quickly become a common laboratory technology with similarly effective environmental application. Foremost to achieve this is a comprehensive understanding of the mechanism and applying this knowledge to achieve high efficiency in a range of bacterial species.

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