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Adaption of Bacteria to Hydrostatic and Osmotic Pressure:
A tale of two sisters

S. Lucas Black

October 2010

A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy
to the
University of Edinburgh
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Abstract

Adaption to environmental stresses is vital for the survival of all organisms living in any environment. Two of the major environmental factors in the deep sea environment are high hydrostatic pressure and high salt concentration. Hydrostatic pressure and osmotic pressure share similarities in their effects on organisms living in the deep sea but this overlap has been little explored.

Major studies from Japan and California over the last 40 years have shown the effects of hydrostatic pressure on bacteria from the deep sea (see [1] for a review). These are complemented by work by Yancey et al. [2] showing that specific solutes accumulated in response to osmotic pressure in fish have the ability to enhance resistance to hydrostatic pressure. However, this work has been done in vitro or with larger organisms and not much is known about the overlap of osmotic and hydrostatic pressure in bacteria.

In this study I investigated the effects of osmotic and hydrostatic pressure on two model organisms: Photobacterium profundum and Escherichia coli. In order to accomplish this task I developed novel imaging equipment which allows for high resolution imaging of bacteria at pressure. I also developed a new method of growing bacteria in 96-well plates at high pressure, which lead to the identification of a hierarchy of genes essential for the growth of E. coli at pressure. I used the same 96-well plate technique to monitor the growth of P. profundum at differing osmotic and hydrostatic pressures. Furthermore I also attempted to analyse the solutes accumulated by different strains of P. profundum in response to osmotic and hydrostatic pressures.
Declaration

Except where otherwise stated, the research undertaken in this thesis was the unaided work of the author. Where the work was done in collaboration with others, a significant contribution was made by the author.

S. Lucas Black
September 2010
Acknowledgements

I’d like to thank Rosalind Allen, my supervisor, she took a chance on me when I work as a research technician and I hope I have lived up to her expectations. Rosalind took an even greater risk in letting me choose my own PhD project, a bet that nearly didn’t pay off, and one I won’t ever repeat with students I may come to supervise. Rosalind has been amazing support to me, I can’t sing her praise enough!

I’d like to thank Bruce Ward, my second supervisor, I was foisted upon him soon after starting my PhD and, even though we’ve not always seen eye to eye, he has been there at times when I’ve needed him and could not have done without his support.

Angela Dawson, Caroline Miles and Hugh Vass have all been instrumental in my PhD. I learnt more about HPLC from Caroline in a week than I had during the previous 2 years, Angela was a constant support for all things in the lab and without Hugh’s insight and experience the work on the pressure cells would never have been the success they now are.

There are many more people who I should thank, but to do so would fill a whole tomb. The people who matter know who they are and I thank them for everything they have done for me.
## Abbreviations

<table>
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<td>%</td>
<td>Percent</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>atc</td>
<td>Anhydrous tetracycline</td>
</tr>
<tr>
<td>BFP</td>
<td>Blue Fluorescent Protein</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan Fluorescent Protein</td>
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<tr>
<td>Chr</td>
<td>Chromosome</td>
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<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
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<tr>
<td>COG</td>
<td>Clusters of Orthologous Groups</td>
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<tr>
<td>DAP</td>
<td>diaminopimelic acid</td>
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<td>dH₂O</td>
<td>Distilled Water</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>ELWD</td>
<td>Extra Long Working Distance</td>
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<td>et al.</td>
<td>et allii (and others)</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>g</td>
<td>Gram</td>
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<td>Gent</td>
<td>Gentamicin</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GPa</td>
<td>Gigapascal</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
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<td>IPTG</td>
<td>Isopropyl thiogalactopyranoside</td>
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<td>Kanamycin</td>
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<td>LB</td>
<td>Laurina Broth</td>
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<td>Liquid Chromatography Mass Spectrometry</td>
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<td>ppm</td>
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<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
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<tr>
<td>RBS</td>
<td>Ribosome Binding Site</td>
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<tr>
<td>Rif</td>
<td>Rifampicin</td>
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<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
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<td>Strp</td>
<td>Streptomycin</td>
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<tr>
<td>Tet</td>
<td>Tetracycline</td>
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<tr>
<td>TMAO</td>
<td>Trimethylamine N-oxide</td>
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<tr>
<td>Abbreviation</td>
<td>Meaning</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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Chapter 1

Introduction

The largest and oldest environment on Earth is the ocean. It is believed that life first began in the ocean some 3.5 billion years ago, however less is known about the bottom of the ocean than is known about the surface of the moon. The reason for this is simple: the technology, skills and enthusiasm to reach and study the moon is, and has always been, far greater than to reach and study the ocean. To make matters worse, the ocean is a planet sized “trash can”; millions of tons of waste and sewerage, thousands of ships, oil rigs and cars have all been dumped into the ocean rather than recycling it or disposing of it on land. Even though the ocean is so poorly treated we rely on it for huge amounts of food, but this too has been exploited. Fish stocks around the world are close to crashing with some species close to extinction. Recently it has been realised at the ocean plays an important role in the geochemical cycles, acting as a carbon skin, effectively slowing global warming [3]. The ocean also play a role in regulating the levels of moisture and thus the climate on a global scale.

The ocean is very large and contains many different habitats and environmental niches. The most widespread environmental conditions in the ocean are low temperature, high pressure and high salt (compared to fresh water and terrestrial environments). The average temperature of the ocean surface is 16°C, which
falls to less than 4°C below 1000m depth. The average depth of the ocean is 3800m making the average pressure close to 38MPa and the average total salt concentration in the ocean is roughly 3.5%. The vast amount of life on this planet lives in these conditions which are totally alien to humans. This leads the question: how are these organisms different to humans and what adaptions allow them to inhabit environments which would kill any human?

Key work on the deep sea has been carried out by labs in California and in Japan since the mid 1950s and in more recent times a more global research community has developed. We now know the types of microbial communities which are present on the ocean floor, the types of metabolism which these communities carry out as well as their interactions with abiotic factors such as deep sea vents.

*P. profundum* is a piezophile (requiring pressure for optimal growth) and a psychrophile (requiring cool temperatures for optimal growth). It was originally isolated from the Sulu Sea in the Philippines, in 1986 by E. F. DeLong [4] and has since become a “workhorse” in the study of bacterial adaption to the deep sea. The majority of this work has come out of the lab of Douglas Bartlett in San Diego, USA. The Bartlett lab has isolated several wild-type isolates of *P. profundum* and have developed basic genetic techniques. The majority of research into *P. profundum* has focused on its response to pressure and temperature. However, one study has investigated the solutes produced by *P. profundum* SS9 in response to pressure and NaCl concentration. Here it was shown that pressure and NaCl concentration had an effect on the types and levels of solute within the cells of *P. profundum*. However, no attempt was made to identify the molecular mechanisms or regulation of production of these solutes. It is known that in the closely related species, *Vibrio cholerae*, the global gene regulator, ToxR/S, is responsible for adaption to different environmental stresses, including changes in osmotic pressure and NaCl concentration. This same global gene regulator has been shown in *P. profundum* to be pressure sensitive, but no work has been done
to link ToxR/S to osmotic regulation.

In this thesis I will discuss the effects of hydrostatic and osmotic pressure on two model organisms, *Photobacterium profundum* and *Escherichia coli* (which has not been subjected to hydrostatic pressure in its evolutionary history), showing that they respond in different ways to the same physical parameters. I will also discuss the new methods for growth and imaging which were developed in order to be able to carry out these studies. In particular I have developed a high-throughput method for growing bacteria at pressure, which has allowed me to study the effects of pressure on the growth of *P. profundum* in higher resolution than previously possible as well as allowing me to quantitatively measure growth of *E. coli* mutants in a screen for pressure sensitivity. In addition, the development of an optical pressure cell, in collaboration with Hugh Vass, for the *in situ* imaging of bacterial samples at biologically relevant pressure has allowed me to further investigate the effects of pressure on these two model organisms. The pressure cell was originally intended to monitor the expression of *toxR/S* at the single cell level in response to changes in both osmotic and hydrostatic pressure. However, *P. profundum* does not express GFP type proteins well making this part of the project all but impossible. Combined with the requirement of GFP for molecular oxygen in order to fold correctly this low expression of GFP resulted in non-fluorescent *P. profundum* cells. Even though the lack of fluorescence was an issue, the pressure cell became very useful in the imaging of *E. coli* samples at a variety of pressures.

In the remainder of this introduction I will discuss relevant research by others in this area.
1.1 Effects of Hydrostatic Pressure on Bacteria

1.1.1 What is Hydrostatic Pressure

Pressure is, like temperature, a universal thermodynamic variable and is important in many areas of research. Physicists, chemists and material scientists study the response of different types of matter to pressure changes and use pressure changes to control the properties of matter [5, 6, 7, 8]. Hydrostatic pressure is also of interest in biological science. For example, pressure can be used to reveal the mechanisms by which protein assemblies and other macromolecular structures form [9, 10, 11, 12, 13, 14]. The effects of pressure within the human body are also studied due to their medical importance: for example, the human knee joints are subjected to increased pressure while walking [15, 16].

Pressure is defined as the perpendicular force applied to a surface, per unit surface area: \( P = \frac{F}{A} \) where \( P \) is pressure, \( F \) is force and \( A \) is area. This relationship can be exemplified by comparing the pressure generated on the ground by a woman in high heels and an elephant. The elephant weighs considerably more than the woman but this force is spread over a larger area; whereas the smaller force of the woman’s weight is focused onto the points of her heels generating a much higher pressure. Thus moderate forces applied over small areas can generate high pressures; a fact that is exploited throughout the research discussed in this thesis.

The units of force are Newton’s (1 Newton = 1 Joule \([\text{J}]\) per meter) and the units of area are \( m^2 \) (meters squared), therefore the units of pressure are Newtons per meter squared or Pascal (Pa). In typical applications one Pa is a very small amount of pressure so Kilo-Pascal (1kPa = 1000Pa) and Mega-Pascal (1MPa = 1000KPa) are more commonly used. Other commonly used units are Bar (1Bar = 99999.9997 Pa) and atmospheres (1atm = 101324.9997 Pa).

For gases pressure, temperature and volume are intrinsically linked by the
ideal gas Law:

\[ PV = nRT \]  \hspace{1cm} (1.1)

where \( P \) is pressure, \( V \) is volume, \( n \) is the number of molecules of gas, \( R \) is the gas constant \((8.314 J/k^{-1}mol^{-1})\) and \( T \) is temperature. This equation describes the fact that as temperature increases in a system at fixed volume the molecules within that system move more quickly, thus exert more force on the walls and therefore increase pressure.

Liquids differ from gases in that their volume changes very little when they are pressurised. For example, for each atmosphere \((0.101\text{MPa})\) increase in pressure the volume of water decreases by only 46.4 parts per million (ppm). For this reason liquids are generally considered to be “incompressible”. The pressure within a liquid is called “hydrostatic pressure”. Because the liquid is incompressible the pressure is the same at any point in a liquid, if no external force is acting upon the liquid.

The pressure difference between the bottom of the ocean and the surface is due to the gravitational forces acting on the water above. The gravitational force on the water column is given by \( F = mg \) where \( g = 9.81\text{m/s}^2 \) and \( m \) is mass. If a water column has area \( A \), and height \( h \) then its mass is \( \rho hA \) where \( \rho \) is the water density. The force due to this weight is \( F = \rho ghA \) and since \( P = F/A \), the hydrostatic pressure is \( P = \rho gh \). The density of water is non-linearly related to temperature. Water is most dense at \( 4^\circ\text{C} \) with density decreasing until boiling point at \( 100^\circ\text{C} \). Below \( 4^\circ\text{C} \) water becomes less dense until forming ice at \( 0^\circ\text{C} \). This is why ice floats which according to NASA may be a major contributing factor to the evolution of life on Earth: the low density water provided an environment free of ice and insulated from cosmic radiation in which life was able to survive during the “Snowball Earth” period which is thought to have occurred ca. 650 million years ago [17].
The effects of hydrostatic pressure on chemical reactions are of vital importance in understanding the effects of pressure on biological systems. In simple terms, a bacterial cell can be considered as a lipid bag containing a thick “soup” in which chemical reactions occur. All chemical reactions have associated with them a “Gibbs free energy change” $\Delta G$. If $\Delta G$ is negative then the reaction is favorable and will progress; reactions with positive $\Delta G$ are unfavorable. The Gibbs free energy change for a given reaction is affected by pressure (assuming a constant temperature) in the following way:

$$\left(\frac{\partial \Delta G}{\partial P}\right)_T = \Delta V$$

(1.2)

The change in $\Delta G$ per unit change in pressure, $\Delta V$, is the “change in the molecular volume” for a given reaction. Therefore any equilibrium reaction which is subjected to an increase in hydrostatic pressure will shift to the side of the equilibrium which has the smallest molecular volume. Note that the molecular volume must be calculated considering not just the reactants and products but also the solvent.

Moderate pressures, less than 100MPa, have various effects on cell physiology. One key effect is that protein-protein and protein-DNA/RNA complexes tend to dissociate. This dissociation is due to the solvent shell contracting and being forced between the sub-units of the complex [18]. For example the ribosome 50S and 30S subunits start to dissociate at 10MPa even though the individual macromolecular subunits remain unchanged [18]. Another effect of moderate pressure is gelation of the phospho-lipid bilayer [19, 18, 20, 21, 22]. This change from a fluid liquid crystal structure to a ridged gel affects membrane proteins as well as the electron transport chain [18]. At very high hydrostatic pressures (>600MPa) the tertiary structure of proteins starts to degrade [18]. Unlike the majority of temperature-denatured proteins, pressure denatured proteins may be able to refold upon release of pressure [23].
Pressure increases also have an effect on the dissociation of acids and bases causing a pH change as pressure in increased [24]. It is for this reason that phosphate buffer is not used in growth media intended for high pressure experiments as its dissociation coefficient is altered with pressure to a large degree, this is not the case for MOPS buffer or HEPES buffer who’s dissociation coefficient is less affected by pressure.

Hydrophobic interactions are thought to be a major contributor to the folding of proteins. As pressure increases the strength of these hydrophobic interactions has to compete with the reduction in $\Delta V$ which would come about due to the solvent gaining access to the hydrophobic core [25]. However this is not the whole picture, the $\Delta V$ is affected more greatly when a cavity within a protein becomes hydrated. The majority of these cavities are formed due to hydrophobic interactions within the protein but can also come about due to other interactions such as hydrophilic interactions.

Hydrogen bonds are also affected by increases in pressure. As pressure increases the strength of the hydrogen bond in also increased [26]. This means that the hydrogen bonds within DNA become strengthened and results in increased stability of DNA at pressure.

1.1.2 High Pressure Environments

The Deep Sea

In comparison to the terrestrial environment, where the pressure ranges from 0.1MPa at sea level to 0.06MPa at the top of Mount Everest, the oceans have a much greater range of pressures (0.1MPa - $\sim$101MPa), to which marine organisms must be adapted in order to survive. At the surface of the ocean, the pressure is roughly 0.1MPa, and increases by 0.01MPa with every 10m depth until the pressure reaches its maximum of 101MPa in the Marianas Trench, where the ocean depth is over 11km. Marine bacteria are, therefore, more
likely to experience severe changes in hydrostatic pressure than their terrestrial counterparts, as they rise and sink with ocean currents.

Organisms which are able to live at elevated hydrostatic pressure are called piezotolerant and those which grow best at elevated hydrostatic pressure are called piezophiles (Figure 1.1). Piezophilic bacteria are of great interest, not only from a basic science point of view, but also for possible biotechnological applications [27, 28, 1, 29, 30, 31].

Figure 1.1: Illustration of the growth phenotypes of piezosensitive, piezotolerant and piezophilic bacteria. Those organisms which are able to grow at pressure are piezotolerant, those which have optimal growth at pressure are piezophilic and those which cannot grow at pressure are piezosensitive [32].

**Sub-Surface Strata**

There is roughly 5-10 billion tons of particulate organic material floating in the oceans, the majority of which is steadily sinking towards the ocean floor as “marine snow”. Some of this marine snow will be subjected to organic degradation by marine organisms (from bacteria to corals and fish) and converted into dissolved organic matter which is “recycled” by marine plants and algae. The remaining marine snow cover the top layers of the ocean floor creating an
environment with 10000-100000 fold higher concentration of organic material than the surrounding water body [33]. The majority of this organic material is degraded by a wide range of Archaea and Bacteria in the upper layers of the ocean floor, but over geological time enough organic material has built up to produce the world’s largest store of carbon \((15000 \times 10^{18} \text{ g C})\) [33, 34]. As in the surrounding water body low temperatures and high hydrostatic pressures are the dominant environmental features in this sediment. In addition, lithostatic pressure is generated by the mass of the sediment above.

### 1.1.3 Pressure-Adapted Organisms

Pressure adaptation is relative: a human is adapted to 0.1MPa and will be killed at pressures above 2.5MPa whereas a sperm whale \((\textit{Physeter macrocephalus})\) can dive to 3000m/30MPa, the greatest depth of any mammal. Fish species such as the Sixgill shark \((\textit{Hexanchus griseus})\) and the Fangtooth \((\textit{Anoplogaster cornuta})\) are often found at depth up to 4500m/45MPa. Invertebrates such as Dumbo octopus \((\textit{Grimpoteuthis sp.})\) and the Bloodybelly comb jelly \((\textit{Lampoctena sp.})\) can be found at depths of 4800m/48MPa and 1000m/10MPa respectively.
It was thought that, due to the lack of oxygen in the deep hypersaline anoxic basins of the Mediterranean Sea (and other deep sea sediments) multicellular organisms could not persist. However Danovaro et al. in 2010 [35] described the first multicellular, exclusively anaerobic organism from deep sea sediments (Sergeeva et al. [36] first discovered these organisms in deep sea sediments but concluded they were contamination from aerobic layers above).

Single-celled life forms such as Archaea and Bacteria are able to grow under anaerobic conditions. These organisms make up the majority of the diversity of the deep sea [37, 1, 30, 31, 33, 34].
1.2 Effects of Osmotic Pressure on Bacteria

1.2.1 What is Osmotic Pressure?

Osmosis is the passage of water through a semi-permeable membrane from a region of low solute concentration to a region of high solute concentration. These solutes can be either ionic or non-ionic molecules. Because of this passage of water the effect of a difference in solute concentration across a membrane is a pressure on that membrane; this is known as osmotic pressure. Osmotic pressure, \( \pi \), is related to the concentration difference of solute across the membrane by the Van t’Hoff law:

\[
\pi = \Delta c R T
\]  

(1.3)

where \( \Delta c \) is the difference in solute concentration in mM, \( R (8.314 J/k^{-1}mol^{-1}) \) is the gas constant and \( T \) is temperature. This means that osmotic pressure is proportional to the concentration of solute molecules within a given volume. Osmotic pressure does not depend on the molecular weight of the solute: a high molecular weight polysaccharide will have the same osmotic potential as a single sugar molecule. For this reason the hydrolysis of starch into glucose causes an increase in osmotic pressure within a cell. The reverse is true for the conversion of glucose into starch.

When a bacterial cell experiences a decrease in external solute concentration an influx of water occurs. This causes an increase in turgor pressure on the cell wall/membrane, which results in the cell swelling and possibly lysing. When the cell experiences an increase in external solute concentration the opposite is true: water leaves the cell and turgor pressure decreases, which can lead to shrinking of the cell and eventually the collapse of the membrane. This is known as plasmolysis.
Figure 1.3: Schematic diagram of the effects of osmosis on the cell. Left, a decrease in external solute concentration causes an influx of water into the cell causing internal pressure (arrows) and possible lysis. Right, an increase in external solute concentration causes water to be lost from the cell causing cell shrinkage and possible plasmolysis.

When the external solute concentration drops the cell often opens up pores in the cell membrane which “dump” the cell contents into the periplasm or surrounding environment to decrease internal osmotic pressure [38]. This method of releasing osmotic pressure is virtually non-selective; meaning that vital as well as non-vital cellular components are lost. In *E. coli* the mechanosensitive channels, MscL and MscS, are responsible for the release of osmotic pressure [39, 40]. In the *P. profundum* genome there is no sequence homology to MscL (Mechanosensitive Channel Large) but there is a homolog of MscS (Mechanosensitive Channel Small) with 57% identity and 74% similarity[41].

There are two main ways in which cells adapt to increases in external solute concentration. Firstly, some cells accumulate high levels of monovalent ions in the cytoplasm, such as K+ Cl- and Na+. This osmo-conforming strategy requires the adaptation of cell components to tolerate high levels of possibly damaging ions and is limited to a select few species in high salt environments such as the halophilic archaea (*Halobacteria*) and anaerobic halophilic bacteria (*Salinibacter rubber*) [42, 43, 44]. The second and more widely adopted method of coping with increasing concentrations of external solutes is to accumulate high levels of so called “compatible solutes”. This strategy has been adopted by a wide variety
of lineages, both prokaryote and eukaryote [45, 46, 47]. The compatible solute strategy involves the synthesis or uptake of small osmotically active molecules which are compatible with normal cell function and can be accumulated to molar concentrations [48, 49, 50].

Figure 1.4: Several common compatible solutes. Glutamate, 2-Hydroxybutyrate and Betaine are found in *P. profundum*.

Because of these limitations the spectrum of compatible solutes used by microorganisms is relatively limited. These include sugars (e.g. trehalose), polyols (e.g. glycerol and glucosylglycerol), free amino acids (e.g. proline and glutamate), derivatized amino acids (betaine and ectoine), quaternary amines and their sulfonium analogues (e.g. glycine betaine, carnitine and dimethylsulfoniopropionate) sulphate esters (e.g. choline-O-sulfate and Trimethylamine N-oxide), and N-acetylated diaminoo acids and small peptides (e.g. N-acetylnornithine and N-acetylglutaminylglutamine amide). The common feature of these molecules is that they are highly soluble and don’t carry a net charge at physiological pH, which would cause changes to metabolic pathways when accumulated at molar concentrations within the cell.
Compatible solutes may function not only in balancing the effects of osmotic pressure across the membrane, but also to stabilize protein complexes and protein conformations. For example in vitro studies of protein folding have shown that TMAO (Trimethylamine n-oxide) is able to re-nature heat denatured proteins [51]. Interestingly, TMAO increases in concentration, in a linear fashion, with depth in marine invertebrates and fish [52], suggesting that it might play a role in piezoprotection. The same could be true for β-hydroxybutyrate in _P. profundum_, as levels of this compound have been shown to increase with pressure as well as with external NaCl concentration [53] (see section 1.4.3 for details).

### 1.2.2 High Salt Environments

**The Deep Sea**

Marine bacteria experience a variety of osmotic conditions in their natural environment since there are variations in the concentrations of NaCl present in the ocean. The majority of the ocean has an average salt concentration of 584 millimolar (mM) with a lower limit of 517mM and an upper limit of 634mM. This is the total salt concentration: the sodium chloride concentration is closer to 180mM, with magnesium chloride, calcium chloride and potassium chloride making up the majority of the other salts found in seawater [54]. Estuaries and coastal waters are particularly prone to changes in osmotic conditions due to the rise and fall of the tides and input of fresh water from rivers. More dramatic than this are the deep sea brine ponds, where “pools” of water saturated with NaCl become segregated from the less salty surrounding water [55]. These pools are formed when the high concentration of NaCl (up to 5 Molar) causes stratification of the more dense saturated water from the less dense surrounding water.
1.3 *Escherichia coli* as a Model Organism

*Escherichia coli* is a gram negative, rod shaped bacterium native to the digestive tracts of mammals, including humans. Because of its ease of culture and long history in the lab *E. coli* is arguably the most studied organism on Earth. This has led to a wealth of knowledge being accumulated for this organism. Large databases of gene and protein function, genomic, proteomic, and metabolomic analysis, as well as a wide variety of genetic tools are all available. However, despite the many years of work over 1755 genes (approximately a third of the annotated open reading frames in the genome) are of unknown function [56]. *E. coli* has probably never, in its evolution, needed to be resistant to pressure: this makes it useful as a model organism.

Hydrostatic pressure is stressful for *E. coli*, therefore as a background to this work it is important to understand the general and specific stress responses in *E. coli*.

1.3.1 General stress response

The general stress response in *E. coli* is induced by several different stresses, including carbon starvation, high or low temperature, high osmolarity and low pH. The general stress response is regulated by the general stress response regulator $\sigma^S$, which is the $\sigma$-factor encoded for by the *rpoS* gene. A $\sigma$-factor is a protein which binds to RNA polymerase, increasing the efficacy with which the polymerase binds to specific promoters. $\sigma^S$ is up-regulated as *E. coli* cells enter stationary phase: this in turn up-regulates many stationary phase genes [57]. Microarray studies have shown that $\sigma^S$ up-regulates 180 and down-regulates 252 ORFs in early stationary phase in *E. coli* [58]. This indicates that approximately 10% of the *E. coli* genome is under the control of $\sigma^S$. Although $\sigma^S$ regulates such a large number of genes specific $\sigma$-factors are also required for survival under specific stress conditions.
1.3.2 Specific stress responses in *E. coli*

Heat shock

The heat shock response is a ubiquitous protective response to cope with heat-induced de-natured proteins, the accumulation of which can be fatal to *E. coli* cells. Over 30 heat shock proteins (HSPs) are involved in the response of *E. coli* to heat-induced protein damage, the majority of which are chaperons, involved in the refolding of de-natured proteins, or are protases, involved in the degradation of damaged proteins. The heat shock response is controlled by the heat shock response regulator $\sigma^{32}$ ($rpoH$).

Upon heat shock, levels of $\sigma^{32}$ are transiently increased, from a low steady state, due to the melting of the $rpoH$ mRNA secondary structure, increasing translation efficiency. $\sigma^{32}$ then binds to the RNA polymerase, which increases its affinity of binding to specific promoters on the genome. This results in the up-regulation of over 30 HSPs including DnaK, DnaJ, GroEL, GroES and $\sigma^{70}$, the “housekeeping” regulator. After 5 minutes, levels of DnaK/J accumulate to such an extent that these proteins start to bind to $\sigma^{32}$, competing for binding to the RNA polymerase. The $\sigma^{32}$ is then degraded resulting in a new steady state, two- to three-fold higher than the pre-heat shock level. The absence of DnaK, DnaJ and GrpE results in increased stability of $\sigma^{32}$ suggesting these proteins are involved in the degradation of $\sigma^{32}$.
Figure 1.5: Wiring diagram of $\sigma^{32}$ regulation. There are three primary modes of regulation as follows: (i) excess free DnaK/J and GroEL/S chaperons directly bind to and inactivate $\sigma^{32}$ forming a feedforward loop; (ii) the FtsH protease degrades $\sigma^{32}$, with chaperons participating in this process, forming a degradation feedback loop; and (iii) temperature directly controls the rate of $\sigma^{32}$ translation, forming an inactivation feedback loop. De-natured proteins titrate chaperons from these regulatory roles, allowing active $\sigma^{32}$ to increase the synthesis of chaperons and protases during conditions where they are needed. Taken from [59]

**Cold Shock**

Cold shock - the sudden shift from 37°C to a much lower temperature, is an environmental shock to which *E. coli* has been exposed to over its evolution. Being part of the mammalian gut flora *E. coli* cells are frequently expelled from the digestive tract (which is 37°C) into the cold environment.

The physiological effects of a sudden cold shock include a phase transition of the cell membrane from liquid-crystal to gel-like (as is the case with increasing pressure), an increase in negative supercoiling of DNA, increased stability of mRNA and defects in translation initiation due to reduced levels of (p)ppGpp and increased levels of the 70S ribosome components.

It is believed that the change in mRNA stability is due to increases in secondary structure of these molecules [60] and the major cold shock protein
CspA is able to act as a chaperon to counter this effect by melting stem-loop structures in the mRNA.

**Acid Stress**

As an enteric bacterium *E. coli* is transmitted through the fecal-oral route passing through the human stomach. *E. coli* has therefore evolved several methods of coping with the extremely low pH (∼pH 1.5-2.5) of the digestive fluids in the stomach before entering the neutral pH of the intestines.

*E. coli* has three “acid-resistance” systems [61, 62]. These systems have optimal performance at differing pH. Their function is to allow the cell to export protons from the cytoplasm into the environment, allowing normal cellular energy-generating functions to continue. The AR2 system, which relies on glutamate, raises the internal pH to 4.2±0.1; the AR3 system, which relies on arginine, raises the internal pH to 4.7±0.1; the AR1 system is still not well understood, but it is known that lysine is essential for its function.

At low pH, and under the regulation of σs and CRP, *E. coli* will up regulate *gadA/gadB* whose gene products cleave glutamate releasing CO₂, consuming 1 proton as part of the AR2 system. The resulting compound, γ-amino butyric acid (GABA) is then exported through the cognate antiporter GadC [63]. In return GadC imports a new molecule of glutamate [64]. In the AR3 system AdiA performs the same as GadA/GadB, with arginine cleavage resulting in agmatine which is exported via AdiC.

The pumping of protons out of the cell causes an electrical potential across the membrane, which if left unchecked would lead to cell death. In the currently accepted model proposed by Foster in 2004 [65], H⁺ dissolves through the cell membrane, AdiC transports arginine (+1 charge) into the cell, this is converted to agmatine (+2) and exported by AdiC, and the resulting CO₂ is able to dissolve out of the cell. At a different rate ClC is importing Cl⁻ ions to counter excess electrical potential while at the same time exporting excess H⁺ ions. The net
result is a positive internal charge as observed by [66] and an increased internal pH. In this way *E. coli* is able to cope with very low pH for several hours.

**Hydrostatic Pressure in *E. coli***

Hydrostatic pressure is widely used as a means of sterilizing food [67] and because of this the response of the food-borne pathogen *E. coli* to pressure has been extensively studied. *E. coli* is also a useful model organism because of the wealth of existing knowledge on its metabolism and gene regulation and the wide variety of genetic tools available.

At relatively low pressures (0.1-30MPa) the cell membrane of *E. coli* undergoes a phase transition from a liquid crystal structure to a gel; this has a drastic affect on the function of membrane proteins, which in turn affects transport, respiration, and metabolism [68]. Also within this pressure range cells undergo a morphological change - they become filamented. It is thought that this filamentation is due to an "SOS" signal pathway becoming activated due to DNA damage [69, 70]. Due to the increased strength of hydrogen bonds at increased pressure it is unlikely that pressure is directly damaging the DNA, it is thought that the damage observed is due to the up-regulation of an endonuclease, Mrr [71]. Within this pressure range *E. coli* is able to gain biomass and replicate its DNA, but at a reduced rate compared to atmospheric pressure [72]. As pressure is increased other cellular functions become compromised: at 50MPa the initiation of DNA synthesis is halted [72]. Cells are able to complete their current round of DNA replication but are unable to initiate new rounds of DNA replication. This suggest the initiation of DNA replication is more sensitive to pressure than DNA replication; something which has been seen in *Photobacterium profundum* SS9 [73]. At 58MPa protein synthesis in *E. coli* becomes halted, with RNA synthesis becoming halted at 78MPa [72].

In the pressure range 90-200MPa *E. coli* viability starts to become affected, depending on growth phase and strain. Evidence from *E. coli* and other bacteria
suggest that loss of viability is due to the loss of membrane integrity [74, 75, 76, 77, 78, 79, 80]; however, cell death follows a similar pattern to that of protein denaturation by hydrostatic pressure [81, 82], suggesting that protein integrity may also be a factor.

Stationary phase *E. coli* cells are more resistant to hydrostatic pressure than exponentially growing cells [81]. This difference has been related to the expression of sigma factor σs [83]. This general stress response regulator regulates over 10% of the *E. coli* genome [84], which makes identification of the specific mechanism for pressure resistance in stationary phase difficult.

The main effect of high hydrostatic pressure (>200MPa) on *E. coli* is loss of integrity of the cell membrane; usually studied through the uptake of vital dyes or the loss of intracellular material [76, 77, 78, 79]. Loss of function of vital proteins such as F1-F0 ATPase and efflux pumps [75, 85], loss of proteins from the cell [79, 86] and dissociation of ribosome subunits [87] have all been shown to be caused by hydrostatic pressure. Since all these effects are fatal to the cell, it is difficult to ascertain which one in particular leads to cell death in *E. coli*.

The use of high hydrostatic pressure (> 600MPa) as a method of food sterilisation is a promising technology because hydrostatic pressure has fewer side effects on taste and texture when compared to thermal or chemical treatments [74, 88].
Figure 1.6: Pressure “time-line” of sensitivity for *E. coli*. The first aspect of *E. coli* physiology to become affected by pressure is the gelation of the cell membrane followed by filamentation, the halting of DNA, protein and RNA synthesis. These are followed by a loss in the ability to gain biomass and finally cell death above 150MPa.

**Osmotic Pressure in *E. coli***

*E. coli* has specific mechanisms to adapt to changes in osmotic pressure. Like most bacteria *E. coli* is able to adapt to a range of osmotic conditions; in a rich media, such as Trypticase soy broth, these range from as low as 0.5% NaCl to as high as 11% NaCl [89, 90]. This begs the question as to how *E. coli* is able to grow over such a wide range of salt concentrations when it has been shown in vitro that even small changes in ion concentration have drastic effects on DNA-protein interactions and gene expression[91].

The key to the ability of *E. coli* to grow at high NaCl concentrations is the accumulation of betaine. *E. coli* can accumulate this compatible solute up to $1.78 \pm 0.05 \ \mu\text{mol/mg protein}^{-1}$ [92]. *E. coli* can take betaine up from the surrounding media via a single-component choline transporter, BetT, [93] as well as synthesizing it *de novo* from choline [94]. BetT is located in the inner
membrane and uses the proton motive force to transport choline, betaine, and betaine aldehyde into the cell. Once choline is in the cell it can be oxidized to betaine aldehyde via BetA. Betaine aldehyde is further oxidized to betaine via BetA/BetB.

1.4 *Photobacterium profundum* SS9 as a Model Organism

*P. profundum* SS9 was first isolated from an amphipod homogenate, at a depth of 2551m in the Sulu Sea in 1986 by Edward F. DeLong [4]. This bacterium belongs to the family *Vibrionaceae*, part of the *Gammaproteobacteria*. *P. profundum* SS9 is a moderate piezophile with optimal growth at 15°C and 28MPa in Marine Broth media [4]. Over the 26 years since its isolation *P. profundum* SS9 has become the model piezophilic microorganism and has been the subject of over a dozen papers.

1.4.1 Pressure Adaptations of the Cell Membrane

Membrane Proteins

Initial investigations focused on the pressure regulation of outer membrane proteins (OMPs). These are highly abundant proteins in bacterial cells which are regulated in response to environmental stimuli. In *P. profundum* SS9 it was discovered that an outer membrane protein, OmpH, was essential for growth at high pressure [95, 96, 97]. It was further discovered that the *ompH* gene is regulated in response to multiple environmental stimuli, including cell density, cAMP and carbon starvation [98]. Another outer membrane protein, OmpL, was found to be highly up-regulated in *P. profundum* SS9 at 0.1MPa, demonstrating that this bacterium able to adapt to both high and low pressure growth [99].
The global gene regulatory protein, ToxR, has been identified as a key factor in the response of *Vibrionaceae* to environmental stimuli [100]. ToxR is a one-component global regulator, it is membrane bound but interacts directly with DNA [101]. In *P. profundum* SS9 ToxR has been shown to be required for adaptive growth at high pressure [102] and is suspected to be involved in sensing of cell membrane topography. ToxR was later shown, via RNA Arbitrarily Primed PCR (RAP-PCR), to regulate several genes in response to pressure [103]. Further to this it was shown that ToxR positively regulates *ompH* and negatively regulates *ompL* in response to pressure [102, 32]. ToxR is discussed in more detail in Section 1.5.

**Membrane Composition**

Membrane composition also plays an important role in pressure adaption of *P. profundum* SS9. It is known that mono- and polyunsaturated fatty acids can increase membrane fluidity at both high pressure and low temperature [19, 1]. For *P. profundum* it has been shown that although growth at pressure increases the percentage of mono- and polyunsaturated fatty acids in the membrane, the genes involved in the production of branched fatty acids are not affected by pressure or temperature [22]. A possible explanation for this was provided by a further study which showed that posttranscriptional regulation plays a role in the production of branched fatty acids in response to pressure [104].

**1.4.2 Genetic Adaptations to Pressure**

The genome of *P. profundum* SS9, which was sequenced in 2005 [41], consists of two circular chromosomes and a mega-plasmid (4.1Mbp, 2.2Mbp and 80Kbp respectively). The presence of two chromosomes is typical of members of the *Vibrionaceae* family with large, stable, plasmids also being found in many strains. Much of Chromosome I (ChrI) comprises “established” genes (those
genes with known function and/or homologues in other organisms) whereas a large proportion of Chromosome II (ChrII) is taken up by open reading frames unique to *P. profundum* (38.6% for ChrII and 18.7% for ChrI). Transposons are also in higher abundance on ChrII than ChrI. The high number of unique ORFs on ChrII has led to the suggestion that ChrII may encode genes required for adaptation to different environments [41]. *P. profundum* SS9 also has the largest number of rRNA operons identified in a bacterium [41, 105] further suggesting that it is adapted to a variety of environments.

![Genome map for P. profundum SS9](image)

**Figure 1.7**: Genome map for *P. profundum* SS9 [41].

Microarray analysis has identified 260 genes which are differentially expressed in *P. profundum* between 0.1MPa and 28MPa. These genes comprise 14 separate Clusters of Orthologous Groups (COG) classifications including amino acid, iron, peptide and carbon transport, amino acid and carbon metabolism, fatty acid biosynthesis, DNA replication and repair and cell wall biosynthesis [41]. In this
study the absolute fluorescence intensity showed ChrI to be more active than ChrII, further supporting the role of ChrII in adaption to multiple environments [41]. Interestingly a complete Stickland pathway (for the fermentation of amino acids) was also identified in *P. profundum* SS9; this is the first occasion this pathway has been identified in a non-obligate anaerobe [41].

The same study showed that *P. profundum* SS9 is a “true piezophile” experiencing stress at 0.1MPa since stress genes, including *htpG*, *dnaK*, *dnaJ* and *groEL*, were up regulated at 0.1MPa compared to 30MPa.

In recent years two other strains of *P. profundum* have been isolated: the type strain DSJ4 [106] and the non-piezophilic 3TCK [41].

<table>
<thead>
<tr>
<th>Feature</th>
<th>ChrI</th>
<th>ChrII</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
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<td>Size(bp)</td>
<td>4,085,304</td>
<td>2,237,951</td>
<td>80,033</td>
</tr>
<tr>
<td>G+C ratio</td>
<td>42%</td>
<td>41%</td>
<td>44%</td>
</tr>
<tr>
<td>Percentage coding</td>
<td>81.3%</td>
<td>78.7%</td>
<td>73.0%</td>
</tr>
<tr>
<td>Number of ORFs</td>
<td>3409</td>
<td>1970</td>
<td>67</td>
</tr>
<tr>
<td>Average ORF size (bp)</td>
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<td>872</td>
</tr>
<tr>
<td>Function unknown</td>
<td>1124</td>
<td>1002</td>
<td>31</td>
</tr>
<tr>
<td>unique to SS9</td>
<td>647</td>
<td>773</td>
<td>26</td>
</tr>
<tr>
<td>not unique</td>
<td>477</td>
<td>229</td>
<td>5</td>
</tr>
<tr>
<td>Transposons</td>
<td>109</td>
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<tr>
<td>Number of rRNA operons</td>
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<td>0</td>
</tr>
<tr>
<td>Number of tRNA genes</td>
<td>145</td>
<td>32</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1.1: Analysis of *P. profundum* SS9 genome sequence [41]
Figure 1.8: Map of the Pacific Ocean showing the locations of *P. profundum* strain isolation. *P. profundum* SS9 was isolated from 2551m depth in the Sulu Sea, Philippines, in 1986, *P. profundum* DSJ4 was isolated from 5110m depth off the west coast of Japan and *P. profundum* 3TCK was isolated from the surface waters of San Diego Bay, USA [4, 106, 41].

Strikingly, there appears to be a strong overlap between the response of *P. profundum* SS9 to hydrostatic and osmotic pressure. Both stimuli have been shown to induce the production of compatible solutes and it is likely that both stimuli also induce gene regulation through the ToxR global regulator (see Section 1.5) [53, 103].

### 1.4.3 Adaptation to Living with Osmotic Pressure

*Photobacterium profundum* copes with changes in osmotic pressure in much the same way as other *gammaproteobacteria*: when challenged with high solute conditions (high salt for example) *P. profundum* will accumulate a variety of different compatible solutes. When challenged by low solute conditions *P. profundum* will “dump” its cytoplasm into the periplasm in order to reduce water uptake and possible lysis. *P. profundum* is thought to do this using an MscS homologue, but this has not been tested.
In 2002 Martin et al. carried out a set of experiments to determine the levels of compatible solutes within the cells of *P. profundum* SS9 as a function of pressure and salt concentration [53]. This study used natural abundance NMR to identify the solutes present in an ethanol cell extract from cultures grown at either 0.1MPa or 28MPa and at either 250mM NaCl or 500mM NaCl in marine broth (a “rich” growth medium). As osmotic pressure increased the levels of compatible solutes (in this case alanine, betaine, glutamate and β-hydroxybutyrate and its oligomer) within the cells also increased, as expected. As hydrostatic pressure increased the levels of compatible solutes also increased - in particular a specific solute (β-hydroxybutyrate) was greatly up-regulated between 0.1MPa and 28MPa (see Figure 1.9).

This study did not investigate whether these solutes were taken up from the Marine Broth medium or synthesized *de novo* by the cells. To determine this, a minimal media lacking β-hydroxybutyrate could be used to test for *de novo* synthesis of this compatible solute. This thesis describes an attempt to do this.
The shallow water strain of \textit{P. profundum} (strain 3TCK), has had its genome partially sequenced. This revealed a set of compatible solute synthesis genes which are lacking in strain SS9. In particular the \textit{ect}ABC pathway imports choline and converts it to ectoine, another major compatible solute used by other \textit{Vibrionaceae} such as \textit{V. cholerae} in response to changing osmotic conditions [107]. This is intriguing because strain 3TCK was isolated from the San Diego bay area where it is likely to encounter changing osmotic conditions due to rain fall and land run off but unlikely to encounter large changes in hydrostatic pressure.

\subsection*{1.4.4 Different Strains of \textit{P. profundum}}

\textit{Photobacterium profundum} DSJ4, the type strain, was isolated at a depth of 5110m in the Ryukyu trench in Japan in 1998 [106]. Although isolated at a greater depth than \textit{P. profundum} SS9, \textit{P. profundum} DSJ4 is considered a midwater
strain due to its lower pressure optimum (10MPa rather than 28MPa); however DSJ4 grows over a much broader range of pressures than *P. profundum* SS9, up to 90MPa. The same microarrays used to identify gene regulation in *P. profundum* SS9 were also used to determine the levels of homology between different strains of *P. profundum*. In this study it was shown that *P. profundum* DSJ4 is 98.7% identical at the 16S rDNA level and has 562 highly divergent or missing genes when compared to *P. profundum* SS9 [108].

*P. profundum* 3TCK was isolated from the surface sediment in San Diego Bay [41] and is considered a shallow water strain as it is not adapted to hydrostatic pressure. Little information has been published about this strain, but more is expected shortly when its genome is published. Using microarray analysis, at the 16S rDNA level *P. profundum* 3TCK is 97.7% identical to *P. profundum* SS9 with 544 divergent or missing genes [108].
Figure 1.10: Phylogenetic tree of *Photobacterium profundum* strains and related species. All strains of *P. profundum* cluster together based on 16S rRNA gene sequence. Strains in bold are piezophiles. Image adapted from [109]
1.5 The ToxR Global Gene Regulator

There are several parallels in the response of *Photobacterium profundum* SS9 to osmotic pressure and hydrostatic pressure. The accumulation of compatible solutes is one such case, as discussed above. Another area of overlap is the regulation of the global gene regulator ToxR.

In *V. cholerae* the ToxR system is used to sense many environmental factors including pH and osmotic conditions [100]. ToxR is a major virulence factor regulating a host of genes including the *ctxAB* operon which encodes the cholera toxin. For this reason the ToxR regulatory system in *V. cholerae* has been well studied. In *P. profundum* the ToxR regulatory system is not linked to virulence but has been shown to be pressure sensitive [27, 103], (Figure 1.11). ToxR is a membrane protein which acts as a ‘one component’ system, binding directly to specific promoter sequences on the genome while still being embedded in the cell membrane. This is a highly unusual regulatory mechanism.

![Figure 1.11: Graphical representation of the effect of pressure on the ToxR/S complex in *P. profundum* SS9. When the complex is functioning (0.1MPa) *ompH* is repressed and *ompL* is induced. It is thought that differences in the turgor of the result in the complex disassembling [102, 32]](image)

Several outer membrane proteins of *P. profundum* and *V. cholerae* are regulated by ToxR in response to changing osmotic conditions. Two of the
outer membrane proteins, OmpU and OmpT, are directly regulated by ToxR in response to osmotic conditions in *V. cholerae*: the *ompU* gene is up-regulated and the *ompT* gene is down-regulated under high salt conditions [100, 110]. In *P. profundum*, OmpU has 41% identity (55% similarity) at the amino acid level and is believed to have the same function at the regulatory level to those for *V. cholerae*; however, *P. profundum* is missing OmpT. The *P. profundum* ToxR protein has 85% identity and 93% similarity as that of *V. cholerae* and ToxR is thought to regulate *ompU* in this bacterium [103, 102].

ToxR has also been shown to regulate OmpH and OmpL in *P. profundum*. These are outer membrane proteins which play an important role in adaptation to hydrostatic pressure [28, 1]. The *ompH* gene is up-regulated by ToxR at high pressure and the *ompL* gene is up-regulated at low pressure. The genes *ompH* and *ompL* are not homologous to *ompU* or *ompT*; however the upstream region of *ompL* has 34 bases identical to that of the upstream region of *ompU*. 
Figure 1.12: Model showing the various regulatory pathways controlled by the ToxR regulator in *V. cholerae*. The ToxRS system up-regulates *ompU* and down-regulates *ompT*. There is down-regulation of motility, up-regulation of cholera toxin and pilus formation also.

1.6 Synthetic Lethal Screening to Identify Genes of Interest

When studying the effect of specific conditions on *E. coli* it is common to look for mutants which are unable to grow under those conditions. These “synthetic lethal” mutants give a good indication of the importance of the disrupted gene in tolerating or adaptation to the experimental condition. Traditionally, deletion mutants have been made via transposon [111] or chemical mutagenesis. Here the mutations produced are at random places on the chromosome; in the case of chemical mutagenesis care must be taken not to produce multiple mutations. Because of the random nature of these methods large libraries of mutants need to be made so that there is a statistical certainty that every gene in the chromosome
had been disrupted at least once. These libraries typically contain over 10,000 mutants, with some containing several times that number. This make the screening of the libraries very labor intensive, with the possible disadvantage that several mutants found may simply be knockouts of the same gene.

### 1.7 High-throughput Growth of Bacteria

Growth of bacteria is normally monitored via one (or more) of three methods: optical density, colony forming units and culture dry weight. Optical density has the advantage that it does perturb the culture being measured. Traditionally, optical density is measured in a spectrometer at a wavelength near 600nm using cuvettes 1cm in thickness (optical density is light absorbance along a path length of 1cm). With the advent of clear 96-well plates and plate reader technology came the ability to measure many cultures at once. While a plate reader is often used only for measurement, some plate readers can maintain constant temperature with shaking, allowing for in situ growth experiments.

![Plate Reader Image](image)

Figure 1.13: Photograph of a BMG Labtech plate reader, the same make and model as used in this study.

The technology behind a plate reader is relatively simple. A motorised stage controls the position of the plate relative to a pair of fixed fiber optic bundles,
which lead from a light source to a detector. In this way, each well of the plate can be positioned so that light shines into the sample from one fiber bundle and the light emerging from the sample is transmitted to a detector by the 2nd bundle. It is important to note that, in order to measure OD correctly, the light source and the detector should be on opposite sides of the sample; in the case of a plate reader this is above and below the plate, so that the sample is within the light path. The amount of light which is absorbed by the sample can then be calculated. A similar approach is used for the measurement of fluorescence and luminescence.

By growing cultures in the plate reader highly time-resolved data can be obtained. Furthermore, it is possible to do very rapid kinetic experiments which last less than a few seconds.

One example of the use of a plate reader to monitor bacterial growth comes from the lab of Uri Alon [112]. Here a library of promoter-GFP fusion plasmids in *E. coli* was created. A plate reader was used to monitor growth and GFP expression during a glucose-lactose diauxic shift experiment: a classic experiment first done in the 1970’s. Alon’s experiments enabled the identification of promoters which had not previously been associated with the glucose-lactose diauxic shift. During this study the growth and fluorescence of over 6000 separate cultures was monitored over a 24 hour time period, resulting in more than 200000 data points. To generate this amount of data would simply not be possible without the plate reader technology.

### 1.8 Microscopy of High Pressure Samples

The use of microscopy to investigate different aspects of biology is ubiquitous. A plethora of microscopic techniques have been developed to investigate different phenomenon. Confocal microscopy is often used to image biofilm formation or to image complex 3D structures in mammalian cells. Fluorescence Recovery
after photobleaching (FRAP) allows quantitative analysis of protein turnover within live cells [113]. Förster resonance energy transfer (FRET) uses a donor photon from the emission of one fluorescent molecule to excite another fluorescent molecule, allowing studies into protein complexes and their binding constants [114, 115]. Total internal reflection fluorescence (TIRF) microscopy use an evanescent light beam to increase the signal to noise ratio for samples within 100nm of the coverslip, a technique which is especially useful for imaging cell membranes [116]. However none of these techniques has previously been used for samples under pressure.

Typically, for microscopic analysis of pressure treated samples, the sample must first be depressurized, then fixed and imaged. The act of depressurizing the samples is a stimulus which can cause changes in the very system being observed: see Figure 1.14 for the effects of fast depressurization. In order to overcome the problem of depressurization artifacts, a microscope pressure cell is needed. These are devices which maintain the sample at the required pressure and temperature while allowing a microscope objective close enough to the sample for imaging. The design of a microscope pressure cells is often a compromise between the need for good optics and high pressures: the better the optical performance the smaller the working distance of the objective and thus the thinner the layer of metal/glass which can be used to maintain the pressure.
Figure 1.14: Electron micrograph of *Methanocaldococcus jannaschii* of cells depressurized over 5 minutes (A) and 1 second (B). Cells depressurized quickly can rupture, spilling the cytoplasm into the surrounding environment and causing cell death. Adapted from [117].

### 1.8.1 Different types of Pressure Cell

Here I will introduce some of the concepts of pressure cell design by highlighting the three main design types for pressure cells. For more detail please see Chapter 3.

**Diamond Anvil Cells**

Diamond anvil cells (DAC) are able to achieve very high pressures, with some being able to reach 10,000MPa (10GPa); however they provide very poor quality images. DAC’s are mostly used for Raman and X-ray spectroscopy techniques. The reason for their poor performance with optical imaging is the thickness and high refractive index of the diamond used for their windows. To alleviate this problem Oger *et al.* [118] developed an asymmetrical DAC, compatible with optical imaging techniques. This “low-pressure diamond anvil cell”, made of Marval 18 steel, uses a standard cut diamond to press against a flat sheet of diamond, separated by a brass gasket, to generate pressures up to 1.4GPa with
a sample volume of just 0.1 µl. This pressure cell is able to generate very high pressures, but the very small sample volume and the poor image quality, due to the diamond, make it unsuitable for the growth of bacteria at pressure or for studying sub-cellular damage caused by very high pressure. Interestingly Oger et al. note that the fluorescence spectra of GFP shifts with pressure at a rate of ∼2.5nm per 500MPa.

**Capillary Pressure Cells**

Capillary pressure cells use a tube of glass (or sometimes quartz) as the sample chamber [119]. This tube is supported with metal surrounds and pressure generated either by pistons compressing the sample within the tube or by an HPLC pump forcing liquid into the tube. The greater the pressure required the thicker the walls of the glass tube need to be, which reduces optical performance. A further reduction in optical performance is brought about due to the curve of the tube itself; as the glass curves light paths are bent causing optical aberrations in the image. Using complex image analysis software it is possible to remove some of these aberrations but more often the glass tube is simply heated and flattened in one section through which images are obtained. This flattening does however weaken the tube and reduce pressure limits.

**Metal Supported Pressure Cells**

Metal supported pressure cells are by far the most common type of pressure cell design; however this is not to say that they are all the same. In some cases pressure is generated between two fixed glass windows, supported by metal surrounds, by an HPLC pump [120]. In another case, two sapphire windows have been forced against each other, in a similar way to diamond anvil cells, to generate pressure [121, 122, 123]. Another method to generate pressure is to use pistons to directly pressurise the sample. This is the type of pressure cell I have developed in this
thesis and I will discuss this design at length in Chapter 3.

### 1.9 Aims and Objectives

The original aim of this PhD was to investigate the possible link between osmotic and hydrostatic pressure. This aim was further broken down into three projects, one large project which was to take the majority of my time and two smaller side projects.

The aim of the main project was to investigate the role of ToxR in the response to osmotic and hydrostatic pressure by building fluorescent reporter strains in *P. profundum* SS9 and imaging these strains at different pressures. This aim meant that a high resolution pressure needed to be developed in order to image these bacteria.

The first of the smaller projects aimed to map the growth of different strains of *P. profundum* in response to both osmotic and hydrostatic pressure.

The second of the smaller projects aimed to isolate and quantify the compatible solutes produced by different strains of *P. profundum* in response to osmotic and hydrostatic pressure.
Chapter 2

Materials & Methods

In this section I will present a series of standard protocols/methods and the material needed to perform those protocols. For details of specific experimental setups please see the appropriate results chapter where modifications or changes to the standard method will be given.

2.1 Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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</thead>
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<td>P. profundum</td>
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</tr>
<tr>
<td>SS9</td>
<td>Wild-type isolate</td>
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</tr>
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<td>3TCK</td>
<td>Wild-type isolate</td>
<td>[41]</td>
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<td>DSJ4</td>
<td>Wild-type isolate</td>
<td>[106]</td>
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<td>SS9R</td>
<td>Spontaneous SS9 mutant, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[97]</td>
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<td>TW30</td>
<td>SS9R ΔtoxRS</td>
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<td>E. coli</td>
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<td>MG1655</td>
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<tr>
<td>DH5α</td>
<td>F- 80dLacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rk&lt;sup&gt;-&lt;/sup&gt; mk&lt;sup&gt;-&lt;/sup&gt;) phoA supE44 λ&lt;sup&gt;-&lt;/sup&gt; thi-1 gyrA96 relA1</td>
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<td>BW25113</td>
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<td>S17(pir)</td>
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Table 2.1: Table of strains used in this work
## 2.2 Bacterial plasmids

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<th>Description</th>
<th>Markers</th>
<th>Reference</th>
</tr>
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<td>p519nGFP</td>
<td>Broad host GFP plasmid P&lt;sub&gt;lac&lt;/sub&gt; and P&lt;sub&gt;npt2&lt;/sub&gt; promoters</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>[129]</td>
</tr>
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</tr>
<tr>
<td>pSET-B tdTomato</td>
<td>tdTomato expression plasmid</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[130]</td>
</tr>
<tr>
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<td>mCherry expression plasmid</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;, Gent&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pBlue-EcFbFp</td>
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<td>Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[137]</td>
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</table>

Table 2.2: Table of plasmids used in this work
2.3 Culture of Bacteria

2.3.1 Culture Media

Luria-Bertani Media
To make 1 litre of Luria-Bertani Broth 10g tryptone, 5g NaCl and 5g yeast extract is dissolved in dH2O to make 1 litre and autoclaved [138]. For solid media 1% w/v agar is added.

MOPS Minimal Media
MOPS minimal medium is made according to Neidhardt et al. 1974 [82] with the addition of varying concentrations of NaCl as required. To make 1 litre of MOPS Minimal Media 10x MOPS Mixture is supplemented with 1.32mM $K_2HPO_4$, 24mM Glucose, 0.1% Casamino Acids and 1:100 Vitamin Mix (final concentration of $2 \mu g$ Biotin, $2 \mu g$ Folic acid, $10 \mu g$ Pyridoxine, $5 \mu g$ Thiamine, $5 \mu g$ Riboflavin, $5 \mu g$ Nicotinic acid, $5 \mu g$ D-Ca-pantothenate, $0.1 \mu g$ Vitamin B12 and $5 \mu g$ p-Aminobenzoic acid.

To make 10X MOPS Minimal Media, 83.72g of MOPS and 7.17g Tricine is added to 300ml dH2O this is set to pH 7.4 with KOH. To this 10ml fresh 0.01M FeSO$_4$$\cdot$7H$_2$O is added followed (in this specific order to avoid a precipitate forming) by 50ml 1.9M NH$_4$Cl, 10ml 0.276M K$_2$SO$_4$, 0.25ml 0.02M CaCl$_2$$\cdot$2H$_2$O, 2.1ml 2.5M MgCl$_2$, 100ml 5M NaCl, 0.2ml Micronutrient mixture (ammonium molybdate, boric acid, cobalt chloride, cupric sulphate, manganese chloride, and zinc sulphate). This is then made up to 1000ml, filter sterilized through a 0.2µm filter and split into 25ml aliquots ready for use.

Marine Broth
To make 1 litre of Marine Broth, 37.4g Marine Broth (Difco) powder is added 500ml dH2O and boiled for one minute. This is then filtered through Whatman 41 grade filter paper to remove precipitate. To the filtered liquid 23g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer is added, the pH
adjusted to pH 7.5 and then made up to 1000ml before being autoclaved. After cooling, sterile glucose is added to a final concentration of 25mM.

Marine Broth has a final composition of: •Peptone 5.0g/l •Yeast Extract 1.0g/l •Ferric Citrate 0.1g/l •Sodium Chloride 19.45g/l •Magnesium Chloride 5.9g/l •Magnesium Sulfate 3.24g/l •Calcium Chloride 1.8g/l •Potassium Chloride 0.55g/l •Sodium Bicarbonate 0.16g/l •Potassium Bromide 0.08g/l •Strontium Chloride 34.0mg/l •Boric Acid 22.0mg/l •Sodium Silicate 4.0mg/l •Sodium Fluoride 2.4mg/l •Ammonium Nitrate 1.6mg/l •Disodium Phosphate 8.0mg/l

For solid media Marine broth was made as above but to 500ml (instead of 1000ml) and after autoclaving mixed with 500ml 2% agar making a total volume of 1000ml. To this sterile glucose was added to a final concentration of 25mM.

ZoBell Media
To make ZoBell Media 5g Peptone, 1g yeast extract and 0.01g FePO₄·4H₂O was dissolved in 227.5ml dH₂O. To this 750ml “stale” sea water, collected from the Crammond shore (Edinburgh) at high tide, was added and the mixture autoclaved [139]. After cooling, sterile glucose is added to a final concentration of 25mM (22.5ml 20% glucose stock solution).

For solid media ZoBell Media was made as above but to 500ml (instead of 1000ml) and after autoclaving mixed 500ml solution containing 15g agar and 7.5g sodium alginate. The sodium alginate was used as a weak selective agent against E. coli in mating experiments.

SOC Media

For recovery after transformation SOC media was used. This is made by mixing together 20g tryptone to 5g yeast extract, 2ml of 5M NaCl, 2.5ml of 1M KCl, 10ml of 1M MgCl₂, 10ml of 1M MgSO₄ and 20ml of 1M glucose. The total volume was made to 1 liter and autoclaved.
2.3.2 Antibiotics

Antibiotics were used as in Table 2.4 for the propagation of plasmids, to select for transformants and to reduce contamination.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Solvent</th>
<th>Stock mg/ml</th>
<th>Final concentration µg/ml</th>
<th>E. coli</th>
<th>P. profundum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>DMSO</td>
<td>50</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Water</td>
<td>25</td>
<td>50</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Water</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Water</td>
<td>500</td>
<td>100</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Water</td>
<td>50</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Ethanol</td>
<td>10</td>
<td>12.5</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50% Ethanol</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Table of antibiotics and concentrations used in this work. All antibiotic stocks are stored at -20°C in 1.5ml aliquot’s until needed.

2.3.3 Growth of Bacterial Cultures

Growth of *E. coli*

**Starter Cultures of *E. coli***. All starter cultures of *E. coli* were diluted directly from -80°C freezer stocks into 5ml fresh LB in a test tube and incubated at 37°C with shaking at 200rpm overnight unless otherwise stated. Antibiotics were used to maintain plasmid propagation as required at the concentrations in Table 2.4 in Section 2.3.2.

**Large Cultures of *E. coli***. For larger cultures of *E. coli* the starter culture is diluted 1:100 or 1:1000 into fresh media and incubated at 37°C with shaking at 200rpm for an appropriate time. Cultures were grown in conical flasks of an appropriate size i.e. for a 50ml culture a 250ml flask was used. This ensured the culture was fully aerated and growing in a uniform manner.

**Growth of *E. coli* in the Plate Reader**. For 96-well plate cultures grown in the plate reader starter plates were diluted directly from -80°C freezer stocks into 200μl fresh LB in starter plate and incubated at 37°C with shaking at 200rpm
overnight. The next day the starter plate is replicated, using a standard 96-well replicator, into a fresh plate containing 200µl fresh LB and placed in the plate reader and incubated at 37°C with shaking at 200rpm. For 24-well or 48-well cultures grown in the plate reader starter cultures of *E. coli* were diluted directly from -80°C freezer stocks into 5ml fresh LB in a test tube and incubated at 37°C with shaking at 200rpm overnight. The next day cultures were diluted 1:100 or 1:1000 into 700-1500µl fresh LB in a plate. This plate was incubated at 37°C with shaking at 200rpm in the plate reader. Typically the OD$_{600nm}$ and/or fluorescence was measured every 10min. Antibiotics were used to maintain plasmid propagation as required at the concentrations in Table 2.4.

**Growth of *P. profundum***

**Starter Cultures of *P. profundum***. To revive from the -80°C stock samples, 20µl was diluted into 5ml Marine Broth in a 15ml falcon tube, this was preformed on ice to avoid heat shocking the cells. The starter culture was then statically incubated at 15°C at 0.1MPa for two roughly 40 hours so that cells are entering late exponential phase (OD0.9-OD1.0). For the propagation of plasmid, antibiotics were used at the concentrations given in Table 2.4.

**Large Cultures of *P. profundum***. To culture large volumes of *P. profundum* the starter culture was diluted 1:100 into fresh media and grown in 50ml flacon tubes, bulbs or bags. The bags and bulbs can be sealed using a heated food bag sealer, this allows them to be placed into the pressure vessel and incubated at pressure. It was important to ensure all air is removed from bags or bulbs before pressure incubation as air compresses causing the bulb or bag to break.

**Conjugation of plasmids into *P. profundum***. In order to insert plasmids into *P. profundum* a tri-parental mating (conjugation) was required. On day 1 a starter culture of *P. profundum* was set up from a freezer stock and incubated at 15°C. On day 3 the starter culture of *P. profundum* was diluted 1:50 into room
temperature marine broth and incubated at 15°C over night (at least 5ml of culture was needed per mating). Also on day 3 starter cultures of *E. coli* helper strains and donor strains were set up and incubated at 37°C over night. On day 4 the *P. profundum* culture was concentrated 50 time and the *E. coli* culture concentrated 5 times giving roughly equivalent numbers of cells in a given volume. The *E. coli* cells were re-suspended in marine broth at room temperature.

Mating experiments were set up as follows:

- Tube 1 - 75 µl *P. profundum* + 75 µl helper strain + 75 µl donor strain
- Tube 2 - 75 µl *P. profundum* + 75 µl helper strain
- Tube 3 - 75 µl *P. profundum* + 75 µl donor strain
- Tube 4 - 75 µl helper strain + 75 µl donor strain

The content of each tube was then spotted into the center of a very dry marine agar plate and left face up with the lid slightly ajar until the liquid was absorbed into the agar. Once dry the plates were sealed in foil and incubated at 20°C for 40 hours.

After 40 hours the bacterial spot was scraped off each plate with a plastic loop and re-suspended in 1ml marine broth. From this 100 µl was taken and spread onto a fresh marine agar plate containing selective antibiotics. These plates were then incubated at 15°C for up to 20 days. If growth was observed on the control plates (those coming from tubes 2-4) no colonies from the mating plate were picked. If the control plates were clear and growth observed on the mating plate these colonies were picked, re-streaked onto fresh marine agar with selective antibiotics. After growth was observed on these plates a single colony was streaked out onto marine agar to ensure a pure culture. From this second purification plate a freezer stock was made.
2.3.4 Storage of Bacterial Cultures

To make a freezer stock a 15ml culture of \textit{P. profundum} was centrifuged at \(\sim 3800\text{G}\), the supernatant removed off and the pellet re-suspended in 700\(\mu\text{l}\) fresh marine broth. To this 300\(\mu\text{l}\) of sterile 100\% glycerol was added. This was then transferred to a 1.5ml cryo-tube and stored in the -80\(^\circ\text{C}\) freezer. To make a freezer stocks of \textit{E. coli} a 5ml culture is centrifuged at \(\sim 3800\text{G}\), the supernatant removed and the pellet re-suspended in 700\(\mu\text{l}\) fresh LB to which 300\(\mu\text{l}\) 100\% glycerol was added. This was then transferred to a sterile 1.5ml cryo-tube and stored in the -80\(^\circ\text{C}\) freezer.

2.3.5 Growth of Bacterial Cultures at High Pressure

Throughout this whole project a large, 3 liter, pressure vessel was used to culture bacteria. This pressure setup consists of a 3 liter steel pressure vessel, rated to 100MPa, a pressure gauge, rated to 100MPa, a hand pump and a safety valve, rated to 70MPa. In order to produce pressure within the pressure vessel the whole system is sealed and the release valve closed. Using a 50ml syringe as a water reservoir water was pumped, by hand into the pressure vessel. The direct pressure was measured on the pressure gauge.
Figure 2.1: Schematic diagram of the 3-liter pressure vessel. Pressure within the pressure vessel is generated via a hand pump, pressure is monitored directly via an in-line pressure gauge and pressure is released via a release valve.

**High Pressure Bulbs & Bags**

For growth in high pressure bulbs the starter culture was diluted 1:100 into 5ml of the appropriate media, the bulb is then heat sealed using a food bag sealer attempting to ensure there was no trapped air. These bulbs are placed into the 3litre pressure vessel and pressurised to the appropriate pressure. For larger cultures (>5ml) sterile plastic bags are used. Briefly, a starter culture was diluted 1:100 into fresh media, placed in a sterile bag and heat sealed using a food bag sealer. Once sealed the bag can be placed in the pressure vessel and grown at the appropriate pressure. Should too large a volume of air be trapped inside the bag or bulb the gas would compress at pressure and possibility cause the bag or bulb to split.
96-Well Plates at High Pressure

In order to overcome limitations in the number of samples able to be grown and measured in pressure experiments it was decided to develop a high throughput method of growing bacteria at high pressure based on 96-well plates. Further details can be found in Chapter 4.

To prepare a pressure resistant 96-well plate a Greiner Bio-One flat bottom 96-well plate was filled with 382µl pre-inoculated growth media when culturing *P. profundum*. When culturing *E. coli* each well was filled with 382µl sterile media and a flame sterile 96-well replicator used to transfer strains from an overnight 96-well plate to the new plate. To cover the surface of the plate a Micro-Amp clear film (Applied Biosystems) was used. After the backing was removed the right-hand edge of the adhesive film was pressed flush with the right-hand edge of the filled 96-well plate. Keeping the left-hand edge of the film roughly 1cm above the plate a roller was used to press the film onto the plate. The use of a roller allowed for greater control and allowed a more pressure to be applied to the film during sealing. To ensure a good contact between the adhesive film and the plate surface the roller was passed over the plate several times with increasing levels of force applied. Once the adhesive film was in place any excess film was trimmed from the plate using a scalpel. A layer of Araldite fast setting epoxy resin was then placed around the edge of the film. Once the Araldite was set the plate could then be pressurised in the 3litre pressure vessel at temperatures up to 42°C and 60MPa for several days. Various different combinations of sealing techniques, seals, adhesives and plates were tried and this combination gave the best results. For details see Section 4.1.


2.3.6 Screening the Keio Collection for Growth at Pressure

Screening the Keio Collection of single, in-frame, deletion mutants was done using the high throughput method described above. On day 1, up to 10 freezer stock plates from the -80°C freezer were allowed to defrost slightly on ice and using a flame sterilized 96-well plate replicator these plates were replicated into a fresh 96-well plate containing 200µl LB supplemented with 25mM glucose and 30µg/ml Kanamycin. These plates were then grown overnight at 37°C with shaking at 200rpm. The next day these plates were then replicated into 382µl fresh LB (25mM glucose and 30µg/ml Kanamycin) and sealed as in Section (2.3.5). These plates were incubated at either 0.1, in a static incubator, or 30 or 50MPa in the 3 liter high pressure vessel for 24 hours. After removing from the pressure vessel and drying the OD_{600nm} for each plate was measured in a BMG Labtech Fluorostar plate reader.

2.4 Cloning

2.4.1 Complementation of TW30

The TW30 strain of P. profundum is a derivative of SS9R lacking a 487bp fragment from the toxR gene. The toxR, toxS and toxR/S fragments of the P. profundum SS9 genome were amplified via PCR using the primers below. These three PCR products were run on 1% agarose gel with 1x TAE buffer and the band of the correct size cut out and the DNA collected using a Qiagen Gel Extraction Kit. The cleaned PCR products were then digested with EcoRI and SpeI (New England Biolabs) in NEB buffer 2 and BSA at 37°C overnight. Shorter digests did not produce fully cut products. The recipient plasmid, pFL190, was also digested over night with EcoRI and SpeI in NEB buffer 2 and BSA. The digested plasmid and PCR products had the digested ends and any protein removed using a Qiagen
PCR Clean Up kit, the DNA was eluted off in molecular grade water. The PCR products were ligated together using NEB quick ligation kit which was left at 4°C overnight to ensure full ligation. The ligation mixture was then transformed (50µl cells to 10µl ligation mixture, 60min on ice, 90 seconds at 42°C, 5min on ice and 1 hour recovery in 1ml SOC Media) into NEB CaCl competent DH5α cells and selected for on LB supplemented with 100µg/ml Streptomycin.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Restriction Site</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxRF</td>
<td>GAATTC</td>
<td>GTACAAATATGATATAGACGCACGAAC</td>
</tr>
<tr>
<td>ToxRR</td>
<td>ACTAGT</td>
<td>TTATTGGCATAGCTTCGAATTATCC</td>
</tr>
<tr>
<td>ToxSF</td>
<td>GAATTC</td>
<td>CAAGCCAAGAGGATAATTCCAAGC</td>
</tr>
<tr>
<td>ToxSR</td>
<td>ACTAGT</td>
<td>GTTAACGGCAATAAGATGTCACTTATG</td>
</tr>
</tbody>
</table>

Table 2.4: Table of DNA primers used in this work

## 2.5 Microscopy

Throughout this project I have used three separate microscopes; a Deltavision epi-fluorescent microscope, a Nikon/Metamorph epi-fluorescent microscope and a BioRad confocal microscope. The Deltavision microscope had six objectives 10x (NA0.4), 20x (NA0.75), 40x (NA1.35), 60x Water (NA1.20) 60x Oil (NA1.40) and 100x Oil (NA1.40). This microscope had filter sets of: 51018, 41017, 86002v1, 52005, 71014, 32014, 41002b, HQ500/20x, HQ535-30m and D436-10x, all purchased from Chroma. Set 41017 was typically be used to image GFP producing cells. The Nikon microscope and the BioRad confocal used the same set of changeable objectives these included the 20xELWD (NA0.40), 60xELWD (NA0.70), 60xWi (NA1.20) and the 100x Oil (NA1.40). The filters on the Nikon included a GFP filter set (Ex470/40nm Em525/50nm Dichroic495LP), RFP set (Ex545/30nm Em610/75nm Dichroic570LP), YFP set (Ex500/20nm Em535/30nm Dichroic525LP) and CFP set (Ex436/10nm Em460/20nm Dichroic450LP).
2.5.1 Bright Field and fluorescence microscopy

For typical imaging of bacterial samples at 0.1MPa bright field or fluorescence microscopy was used. Bacterial samples were grown overnight at the appropriate pressure and temperature; should antibiotic be required (for the propagation of plasmids etc.) it was added as in Table 2.4.

Slides and cover slips were cleaned in acetone and allowed to dry on the bench, ensuring the removal of artifacts from the optical surfaces.

Once dry 10µl of culture was added to the slide and a cover slip placed on top. For long imaging sessions or when transporting slides outside the lab a thin layer of nail polish was applied around the edge of the coverslip adhering it to the slide. This reduced evaporation from the slide and kept bacteria contained during transport.

For bright field and fluorescent microscopy a Nikon TI-U inverted microscope with a Sony CoolSnap HQ camera was used. Before imaging the objective would be fitted and the microscope Kӧhler adjusted. Using Metamorph software the illumination channels, exposure times and CCD bin settings were selected. Scale bars and other image alterations were also carried out using Metamorph software.

2.5.2 Confocal Microscopy

2.5.3 High Pressure Microscopy

Bacterial cultures were grown overnight or to OD\(_{(600\text{nm})}\) 1.0 in the appropriate media, at the required temperature and pressure. These cultures were diluted 1:1000 into fresh media and 100µl added to the pressure cell being used. The piston was placed firmly into the pressure cell and tightened by adjusting the screw on top of the piston. Once secure excess culture was mopped up with absorbent paper. The pressure cell and piston were then placed in the ram and securely screwed into place by hand, followed by further tightening using a pair
of tweezers. Pressure could then be applied to the pressure cell via a pressure pump.

Temperature was controlled via a brass ring fitted with heater elements attached to a temperature controller (Lakeshore 331 Temperature Controller). Temperature was monitored via a thermocouple cable which fed directly into the temperature controller.

To cool the pressure cell the brass ring, and temperature controller was fitted to the ram as described above with the addition of cold nitrogen gas being flown around the brass ring. Liquid nitrogen was heated slightly with two resistors attached to a power supply, the evaporated gas was channeled through a rubber tube, warming along the way, to flow over the brass ring around the pressure cell. This method of cooling would cool the pressure cell to below 0°C therefore a current was passed through the heater elements within the brass ring. Temperature was stable and controlled to within 0.1°C.

2.6 High Performance Liquid Chromatography

2.6.1 Preparation of HPLC Samples

Bacterial cultures were grown in the appropriate media at the required pressure in 5ml volumes. Once at OD_{600nm} 0.8-1.0 cultures were harvested at 4500rpm (∼3800G), the supernatant was discarded and the pellet was frozen to -80°C and lyophilized overnight. The weight of the dry pellet was recoded. To extract the solutes 1ml 70% ethanol was added and the pellet re-suspended. The mixture was sonicated for 10min in a sonic water bath and then centrifuged at 13000rpm (∼10000G) for 10min. The supernatant was then recovered for further processing. The pellet was treated to another 2 rounds of sonication and centrifugation, the recovered supernatant was pooled from all three extractions. The supernatant was rotary evaporated, removing the bulk ethanol. Once reduced to less than
500\(\mu\)l the supernatant was frozen to -80\(^\circ\)C and lyophilized overnight.

The extracted solutes were re-suspended in 1ml deionized water and vortexed. From this extraction solution 50\(\mu\)l was added to 950\(\mu\)l acetonitrile. If a precipitate was produced the extraction solution was diluted 1:10 and 50\(\mu\)l of the dilute solution was added to 950\(\mu\)l fresh acetonitrile. The final mixture was filtered through a 0.45\(\mu\)m PTFE filter in preparation for HPLC analysis.

### 2.6.2 Forward Phase Chromatography of Bacterial Extracts

To analyse the extracted solutes High Performance Liquid Chromatography (HPLC) was used. A Dinoex UltiMate 3000 HPLC system with a GromSil 80 Amino-1 PR column attached was run in a forward phase manner (water is the 'strong' solvent). After injecting 20\(\mu\)l of sample onto the column the mobile phase was kept at 95% acetonitrile for 2min, then over 20min the acetonitrile concentration was reduced to 60% where it was kept for an additional 3min. The acetonitrile concentration was restored to 95% and the column equilibrated for 10min prior to the next sample being run. Total run time was 35min. Absorption was measured at 210nm for the detection of solute peaks.

For initial samples peaks were collected on a FoxyJr fraction collector using peak detection mode. These fractions were allowed to dry in a fume hood and re-suspended in HPLC grade water prior to being sent for identification by mass spectrometry in the School of Chemistry.

Protein quantification was used to standardize all samples allowing samples to be compared with one another. To quantify the levels of protein within the cell pellet a Nano-Orange assay was used according to the manufacturers instructions. Briefly the cell pellet was dried and re-suspended in 500\(\mu\)l dH\(_2\)O, 1\(\mu\)l was then added to 199\(\mu\)l 1X nano-orange reagent and incubated at 95\(^\circ\)C for 1 hour. After cooling to room temperature the reaction mixture was transferred to a 96-well
plate and the fluorescence measured at 570nm with excitation at 470nm. This was compared to a standard curve of protein concentration performed on known amounts of BSA from 100ng/ml to 10µg/ml.
Chapter 3

Development and Testing of an Optical Pressure Cell

3.1 Introduction

The drive for the development of technology and equipment for the study of hydrostatic pressure can, broadly speaking, be divided into two groupings: 1) those wishing to study the effects of moderate hydrostatic pressure (~200MPa) on environmental samples and 2) those wishing to study the effects of high hydrostatic pressure (>600MPa) for food sterilisation. Focusing on the development of optical equipment, the study of high hydrostatic pressure has lead to the development of diamond anvil pressure cells [140]. These cells are capable of several hundred gigapascal (GPa) of pressure [141] and are widely used with spectroscopy techniques [140]. However diamond anvil cells are not suited to microscopy techniques (with the exception of [118]) due to the thickness and optical properties of the diamond used.

For the study of samples at moderate hydrostatic pressure various different designs of pressure cells for microscopic use have been developed. Some of these have been summarized in Table 3.1. In general each pressure cell is unique to the
task it was designed for; there is no universal design for this type of pressure cell.

The design of an optical microscopic pressure cell (hereafter referred to as pressure cell) necessitates that the cell be robust enough to hold the pressure required; ideally there is a safety factor built into the pressure cell whereby it can hold greater pressure than required. This translates into using thick walls to withstand the pressure required within the sample chamber. However the pressure cell must also have at least one window for observations. For microscopic observations, as a general rule, the greater the magnification the closer the objective needs to be to the sample. The distance between the objective and the sample is known as the working distance of an objective; in general the smaller the working distance the greater the magnification. As a rule of thumb the greater the magnification of an objective the greater its Numerical Aperture (NA): the greater the NA the higher the resolving power of an objective. The NA is a measure of the optical performance of an objective, given by \( NA = n \sin \theta \) where \( n \) is the refractive index of the immersion medium and \( \theta \) is half the angle of the cone of light entering or exiting the lens. By reducing the distance between the sample and the objective the pressure cell becomes weaker and therefore the maximum pressure obtainable is reduced (see Section 3.1.1). A compromise therefore has to be struck between maximum pressure and optical performance.

In this chapter I will describe the two pressure cells (Cell 1 and 2) developed previously in our department by Eva Herzig, Paul Clegg and Hugh Vass for the imaging of colloidal suspensions while at pressure. I will then compare and contrast their results with three pressure cells (Cell 3, 4 and 5) which I have developed, in collaboration with Hugh Vass, for the imaging of live bacteria at pressure.

By using the previous designs of pressure cell as a starting point I aimed to develop a modular system which is capable of imaging at over a wide range of pressures on a wide range of microscope equipment. All the pressure cells are interchangeable for multiple applications.
Table 3.1: Technical properties of a number of high-pressure microscope cells developed for biological and soft-matter applications.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Application</th>
<th>Sample volume</th>
<th>Max Pressure</th>
<th>Optical properties</th>
<th>Pressurization</th>
<th>Special features</th>
</tr>
</thead>
<tbody>
<tr>
<td>[118]</td>
<td>Microbiology</td>
<td>0.1µl</td>
<td>1.4GPa</td>
<td>Diamond window 250-600µm thick, 4mm diam. 20X objective</td>
<td>Gas-filled ram</td>
<td>Diamond anvil cell Temp range to 300°C</td>
</tr>
<tr>
<td>[142]</td>
<td>Microbiology</td>
<td>25µl</td>
<td>700MPa</td>
<td>Sapphire window 5mm thick, 10mm diam. 20X objective</td>
<td>Hand pump</td>
<td></td>
</tr>
<tr>
<td>[143]</td>
<td>Polymer physics</td>
<td>~300µl</td>
<td>300MPa</td>
<td>sapphire or diamond window ~2mm thick, ~5mm diam, WD 12mm 10X objective</td>
<td>Hand pump</td>
<td>Polarization microscopy Temp range -40-270°C</td>
</tr>
<tr>
<td>[144]</td>
<td>Liquid crystals</td>
<td></td>
<td>300MPa</td>
<td>Sapphire window 5mm diam., WD 3.5mm 20X objective</td>
<td>Hand pump</td>
<td>Temp range 20-250°C</td>
</tr>
<tr>
<td>[121, 122, 123]</td>
<td>Cell Biology</td>
<td>3.5µl</td>
<td>300MPa</td>
<td>Sapphire window 2.3mm thick, WD 1.5mm. 20X or 40X objective</td>
<td></td>
<td>Fluorescence and transmitted light</td>
</tr>
<tr>
<td>[145]</td>
<td>Biophysics</td>
<td>200 µl</td>
<td>200MPa</td>
<td>Optical glass window 1.5mm diam., WD 6mm 40X objective</td>
<td>Hand pump</td>
<td></td>
</tr>
<tr>
<td>[120]</td>
<td>Cell biology</td>
<td>100µl</td>
<td>100MPa</td>
<td>Pyrex glass window, 2mm thick, 2mm diam., WD 3.8mm 20X or 40X objective</td>
<td>HPLC pump</td>
<td>Continuous flow Temp range 2-80°C</td>
</tr>
<tr>
<td>[146]</td>
<td>Cell Biology</td>
<td>75µl</td>
<td>80MPa</td>
<td>Strain-free glass 1.75mm thick, 3mm diam., WD 2.3mm 40X objective</td>
<td>Hand pump</td>
<td>Polarization microscopy</td>
</tr>
<tr>
<td>[119]</td>
<td>Biological cells</td>
<td>~1µl</td>
<td>100MPa</td>
<td>Image through wall of glass capillary 0.34 or 0.5mm thickness 10X objective</td>
<td>Hand pump</td>
<td>Fluorescence microscopy Glass capillary tube</td>
</tr>
<tr>
<td>[147, 148]</td>
<td>Colloid physics</td>
<td>190µl</td>
<td>40MPa</td>
<td>Diamond window 1mm thick, 2.8mm diam, NA 0.25 10X objective</td>
<td>HPLC pump</td>
<td>Continuous flow Temp range 20-450°C</td>
</tr>
<tr>
<td>[149]</td>
<td>Cell Biology</td>
<td>100µl</td>
<td>15MPa</td>
<td>Glass coverslip 0.15mm thick, 1mm diam. 40X objective</td>
<td>HPLC</td>
<td>Continuous flow Fluorescence and transmitted</td>
</tr>
<tr>
<td>[150]</td>
<td>Cell Biology</td>
<td>9ml</td>
<td>10MPa</td>
<td>Sapphire window 2mm thick, 30mm diam. 40X objective</td>
<td>HPLC flow cell</td>
<td>Electrical stimulation of cells</td>
</tr>
<tr>
<td>[151]</td>
<td>Cell Biology</td>
<td>~1ml</td>
<td>7MPa</td>
<td>Glass coverslip 0.2mm thick, 1mm diam. 40X, 1.3NA objective (fluorescence)</td>
<td>HPLC pump</td>
<td>Fluorescence and transmitted</td>
</tr>
</tbody>
</table>
3.1.1 Pressure Cell Design Features

The design of the pressure cell determines the pressures which can be obtained safely within the sample chamber. In particular the region around the window support is the weakest point within a pressure cell. To produce a pressure cell which can sustain a high sample chamber pressure the thickness of the window support can be increased (Figure 3.1A). However this prevents the objective approaching close to the window, thus reducing the magnification of the objective that can be used. Another way to increase maximum pressure is to reduce the window aperture of the pressure cell. This too has a detrimental effect on the optical properties of the pressure cell, since a small aperture can clip the cone of light entering the objective and cut down the resolving power of that objective below its optimum. It was for this reason that, in the work done by Herzig, Vass and Clegg, Cell 2 had a cone section removed from the window aperture (see Section 3.2). This design feature has been carried through to Cell 3 where the edges of the window aperture are ground at an angle allowing the full NA of 0.70 for the 60x ELWD objective to be obtained.

Another design feature is to round the edges of the metal within the sample chamber. This has the effect of spreading the stresses over a greater surface area around the corners rather than focusing the stresses on a single point (Figure 3.1B).

3.1.2 Stresses on the Pressure Cell

Physical Stresses

In order to design a safe optical pressure cell it is important to understand the stresses acting at different points within the pressure cell. The classical method of calculating the stresses within a pressure vessel is to treat it as a thick walled cylinder using Lame’s equations . The stresses on such a cylinder act in the radial, axial and tangential (hoop stress) directions (See Figure 3.2).
The axial component ($\sigma_1$) of the stress of most importance, as this is likely to cause failure of the pressure cell window. The axial stress is given by:

$$\sigma_1 = \frac{P_i r_i^2 - P_o r_o^2}{r_o^2 - r_i^2}$$

(3.1)

where $\sigma_1 =$ axial stress, $P_i =$ internal pressure, $P_o =$ external pressure, $r_i =$ internal radius and $r_o =$ external radius.

The tangential stress ($\sigma_2$) component is given by:

$$\sigma_2 = \frac{P_i r_i^2 - P_o r_o^2}{r_o^2 - r_i^2} + \frac{r_i^2 r_o^2 (P_o - P_i)}{r^2(r_o^2 - r_i^2)}$$

(3.2)

where $\sigma_2 =$ stress in tangential direction at radial distance $r$ in the pressure.
Figure 3.2: Directional Stresses within a thick walled pressure vessel showing the axial stress ($\sigma_1$), the tangential stress ($\sigma_2$) and the radial stress ($\sigma_3$).

The radial stress ($\sigma_3$) at radial distance $r$ in the wall of a thick walled pressure vessel is given by:

$$\sigma_3 = \frac{P_i r_i^2 - P_o r_o^2}{r_o^2 - r_i^2} - \frac{r_i^2 r_o^2 (P_o - P_i)}{r^2 (r_o^2 - r_i^2)} \quad (3.3)$$

where $\sigma_3 =$ stress in radial direction. $\sigma_3$ is greatest when $r=r_i$ i.e. at the inner surface of the wall.

However, an optical pressure cell is not identical to a closed end pressure vessel, as described by Lame’s equation’s, because the window has a significant effect on the pressure tolerance of the pressure cell. Fortunately the stresses in a windowed pressure cell can be calculated using equations similar to the classical thick walled pressure vessel equations [146]. Each of the following quantities (3.4, 3.5, 3.6) has been calculated for each pressure cell described in this chapter using the dimensions in Table 3.2.

The material tensile yield strength $\sigma_{vp}$ is most directly related to the thick walled cylinder calculations above, using the ratio of the internal radius and
external radius \( (k) \) as the primary factor in determining the pressure at the surface of the sample chamber wall [146]. Unlike Equation 3.3 which allows one to calculate stress at multiple points within the wall equation 3.4 assumes one is only interested in the stress at the inner surface of the wall. \( \sigma_v p \) is given by:

\[
\sigma_v p = P_c \frac{k^2}{k^1 - 1}
\]

where \( P_c \) is the pressure within the sample chamber and \( k \) is the ratio of outside to inside diameter. However if \( k \geq 3 \) there is little advantage gained from this added metal thickness: for Cells 1-3 \( k \geq 7.6 \) making deformation unlikely before the window support yields.

The window support area is subject to greater stress \( (\sigma_s) \) than the sample chamber pressure as a whole [146] because the window port region has a smaller radius than the rest of the sample chamber. This calculation is derived from the axial stress as shown in Equation 3.1 and is given by:

\[
\sigma_s = \sigma_c \frac{r_\omega^2}{r_\omega^2 - r_p^2}
\]

where \( r_\omega \) is the radius of the window and \( r_p \) is the radius of the window aperture (port). For Cell 1 \( \sigma_s = 1333 \text{MPa at } \sigma_c = 1000 \text{MPa}, \) the ultimate tensile strength of TiMetal 550 is \( \sim 1200 \text{MPa}. \) For Cell 2 \( \sigma_s = 933 \text{MPa at } \sigma_c = 700 \text{MPa} \) and for Cell 3 \( \sigma_s = 93 \text{MPa at } \sigma_c = 70 \text{MPa}. \) These are well within the ultimate tensile strength of TiMetal 550; see Figure 3.3.

Finally, shear stress \( (\tau) \) is the primary stress on the cylinder ends, thus the main stress on the pressure cell window support. Shear stress is defined as a stress which is applied parallel or tangential to a face of a material, as opposed to a normal stress which is applied perpendicularly. Shear stress determines the minimum metal thickness required to support the window at the desired pressure [146]. Shear stress is given by:
\[ \tau = \frac{Pcr}{2h} \]  

(3.6)

Where \( r \) is the radius of the end section and \( h \) is the thickness of the end section.

As a guideline for titanium alloys the ultimate shear strength is roughly 0.65 times the ultimate tensile strength [152]. TiMetal 550 has an ultimate tensile strength of \( \sim 1200 \text{MPa} \) and the ultimate shear strength is roughly \( 780 \text{MPa} \). For Cells 1-3 the shear pressure applied to the surface of the window support at 1000MPa, 700MPa and 70MPa are 525MPa, 403MPa and 107MPa respectively.

In all cases the pressure cell is able to hold a greater pressure than at which it is used: this gives a safety margin should an accidental over pressurisation occur. When designing a pressure cell a safe maximum pressure should be considered to be roughly 1.5 times the maximum working pressure, this is easily the case for Cells 2 and 3 meaning there is little chance these cells would fail under normal use. Cell 1 does not meet the 1.5 times safety pressure so is limited to a maximum pressure of 700MPa.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Height</th>
<th>Internal Diameter</th>
<th>External Diameter</th>
<th>Window Support</th>
<th>Window Thickness</th>
<th>Window Aperture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>13.5</td>
<td>35</td>
<td>4.2</td>
<td>4.0</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Cell 2</td>
<td>13.5</td>
<td>35</td>
<td>4.5</td>
<td>4.0</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Cell 3</td>
<td>10.5</td>
<td>35</td>
<td>4.5</td>
<td>1.5</td>
<td>0.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 3.2: Dimensions for pressure cells 1-3 as used in equations 3.4, 3.5 & 3.6.

Optical Stresses

As pressure inside the sample chamber is increased the material of the window will flex slightly. This flexing will cause the window to become a lens, bending the light path and degrading optical performance of the window at pressure. The amount of flex and in turn the amount of optical aberration is dependent on the
Figure 3.3: Pressure calculations based on Cells 1-3 built using TiMetal 550 with a yield pressure of 1070MPa and a fail pressure of 1200MPa. For Cell 1 & 2 the safe working pressure is below 800MPa, at which point the window support could yield. For Cell 3 the safe working pressure is below 650MPa, at which point the shear stress is greater than the metal yield. In all cases $\delta p$ is measured at on the sample chamber’s internal wall and will decrease through the metal.

Pressure during imaging, the refractive index of the window material, the pressure resistance of the window material and the window area free of support.

Optical path difference $\delta$ is a measurement of the change in optical path length (in this case) between the pressure cell window and the objective due to the flex of the window while at pressure [153]. The greater the value of $\delta$ the higher the lensing effect and thus the optical abberation.

$$\delta = 8.89 \times 10^{-3} \left\{ (n - 1) \left( \frac{\Delta P}{E} \right)^2 \left( \frac{d}{h} \right)^5 d \right\} \quad (3.7)$$
where \( n \) is the index of refraction, \( \Delta P \) is the pressure differential, \( E \) is the window Young’s modulus, \( d \) is the window diameter and \( h \) is the window thickness. For Cell 1 and 2 the diamond window has Young’s modulus of 1220GPa, an exposed diameter of 1.5mm and a thickness of 0.5mm. For Cell 3 the quartz window has a Young’s modulus of 71.7GPa, a diameter of 1.5mm and a thickness of 0.5mm. And for Cell 4 the quartz window has a Young’s modulus of 71.7GPa, a diameter of 0.5mm and a thickness of 0.2mm.

### 3.2 A Pressure Cell for Imaging Colloids

The pressure cell system (cells 1 & 2) previously developed by Hugh Vass, Eva Herzig and Paul Clegg in the School of Physics was designed to study colloid phase transitions in response to temperature and pressure [154]. This system was required to hold pressures up to 150MPa, have temperature control (ambient to 45±0.1°C), be chemically resistant and allow for fast and deep pressure quenches. Direct pressurisation of the sample was also required in order to keep the sample separate from the pressurizing medium.

Specific details of the design, construction and testing of the pressure cells of...
Figure 3.5: Calculated Optical path difference for Cells 1-4. This shows that cells 1 & 2 have identical optical performance, that cell 3 has the greatest distortion due to flexing of the window and that cell 4 has the least distortion within its pressure range.

these specifications can be found in Reference [154]. These two pressure cells fit an Extra Long Working Distance (ELWD) 20x objective with a working distance of 4mm. Each cell is made of TiMetal 550 titanium alloy and consists of two parts: the pressure cell itself and the sample piston. The pressure cells and the piston have 0.5mm thick diamond windows fitted using a chemically resistant adhesive (MBond GA-61), which is set at 150° C, with a Poulter type seal.

In addition to the pressure cell and piston, the system includes a pressurizing ram and ram piston. The ram is attached to a pressure pump via a hydraulic hose and pressure gauge. Pressure is generated by pumping hydraulic fluid into the ram, forcing the ram piston down onto the pressure cell piston thus creating
pressure within the sample chamber. The large difference in piston areas acts as a pressure multiplier, so that the pressure measured on the gauge is approximately 20-fold lower than the actual pressure in the sample chamber (see Section 3.1.2).

The first of these pressure cells, known as Cell 1, (Figure 3.6A) has a maximum working pressure of 500MPa. This cell has window support of thickness 4mm surrounding a 0.5mm thick, 3mm diameter diamond window with a 1.5mm aperture set at 90° to the window surface. Cell 1 has a solid sample piston, also made of TiMetal 550, which uses two sealing rings: one of PTFE and the
other of phosphorbronze, allowing sealing at both moderate and high pressures. The use of PTFE as a sealing ring had specific advantages: it removed the need to use greased rubber seals, ensuring samples free of grease contamination while maintaining pressures from 0.1MPa to 300MPa. This piston fits the 4.2mm bore of the sample chamber. Cell 1 had an effective Numerical Aperture (NA) of 0.17, well below the NA value of 0.45 corresponding to the 20x ELWD objective under optimal conditions.

In order to improve the optical performance, a second pressure cell was built: Cell 2, (Figure 3.6B). In this cell the angle of the window aperture was opened up, achieving an NA of 0.34, which resulted in improved optical quality for this pressure cell [154]. However this improvement came at the cost of a reduction in the maximum working pressure obtainable which was 150MPa for this cell.

Some experiments carried out by Herzig, Vass & Clegg [154] required transmitted light microscopy. To allow this, a hollow ram was developed, along with a hollow sample piston (Figure 3.6D). This allowed light from the microscope condenser to pass through a diamond window in the piston and into the sample chamber.

Another development in pressure cell design achieved by Herzig, Vass & Clegg [154] was the incorporation of a solenoid valve between the pressurizing pump and the pressure cell. This valve allowed for fast (<0.1 second) and deep pressure quenches by dumping the hydraulic fluid in the ram into a reservoir attached to the ram. The solenoid valve had the added advantage of stabilizing pressure within the pressure cell over long periods of time.

3.3 A Pressure Cell for Imaging Bacteria

The imaging of microbiological samples requires the use of high magnification, high numerical aperture (NA) optics to resolve bacterial morphologies and subcellular structures. Achieving such optical qualities while maintaining
hydrostatic pressure was a major challenge.

My work required a pressure cell that could be used with at least a 60x objective with a high NA (around 1.00). Accurate and stable temperature control from 4°C to 42°C with a pressure range from 0.1MPa to 100MPa were also required. The pressure had to be stable over long periods of time to allow bacterial samples to grow at pressure for up to several days. In addition to this both transmitted and fluorescent light microscopy were required.

3.3.1 Cell 3

Using the design of Cells 1 and 2 as a starting point, I designed a new pressure cell able to fit a 60x ELWD objective, with an NA of 0.70 and a minimum working distance of 1.5-2.0mm. This new pressure cell, Cell 3, has a metal thickness around the window of 1.5mm: drastically reducing the maximum pressure obtainable (See Section 3.1.2). To overcome this limitation a heat treatment was developed by Hugh Vass which added strength to the TiMetal 550 titanium alloy. This treatment, based on the method for producing titanium springs, increases tensile strength by an estimated 10% (tensile strength of TiMetal 550 is 1200MPa prior to treatment). The heat treatment proceeded as follows: once the pressure cell had been machined to a rough shape, ground and polished to the exact specifications, it was then heated to 900°C for 1 hour, leading to a disordered “solution” configuration of the atoms within the alloy. The cell was then oil quenched, cooling the metal rapidly and setting the atomic structure of the alloy in place. A final heat treatment of 500°C for 24 hours tempered the metal, increasing its tensile strength. This heat treatment had the additional advantage of darkening the metal, reducing reflections and “stray” light within the pressure while imaging.

Cell 3 has a window of 4.5mm thick quartz. By changing the window material from diamond to quartz, the refractive index of the window material was more
closely matched to that of the glass of the objective. Diamond has a refractive index of 2.41, quartz 1.46, glass 1.51 and air 1.00. As light passes through the boundaries between materials of different refractive indices it is refracted by an amount that is dependent on the difference in refractive indices, so by using the quartz window the loss of light from my samples was reduced and improved imaging over Cells 1 and 2 was obtained.

In order to control temperature below room temperature a system based on the flow of cold nitrogen gas and a heater element was used. Briefly, a tank of liquid nitrogen was heated slightly by two resistors which were lowered into the liquid; the cold evaporated gas then passed through a tube and warmed slightly. This gas was then passed around the heater elements of a bronze heating ring fitted around the pressure cell ram causing the pressure cell to cool. Temperature was finely controlled by applying a current to the heater elements placed around the ram causing it to warm the pressure cell. It was this balance between cooling and heating that allows the pressure cell to be cooled below ambient temperature and allows very tight temperature control. In my experiments temperature was controlled from 4°C to 37°C±0.1°C.

![Figure 3.7: To scale drawing of pressure cell 3. This pressure cell has a shallower metal thickness around the window allowing a 60x ELWD objective to approach close enough to the sample chamber to achieve its required working distance of 2.1-1.5mm.](image)
3.3.2 Cell 4

Following the development of Cell 3 a fourth pressure cell was developed (Cell 4) to fit a water immersion (Wi) objective. This objective has a much higher NA than the ELWD objective, allowing for improved image quality. For this pressure cell the window was moved from the internal ledge and replaced by a revolutionary new design, the window aperture was reduced from 1.5mm to 0.5mm. This window design had a breaking pressure of 120MPa, but allowed a 100x oil immersion objective (NA 1.40) close enough to image within the sample chamber. Cell 4 was also made of a different titanium alloy, TiMetal 551, which has a greater ultimate tensile strength (by roughly 20%) than TiMetal 550 (1450MPa for 551 and 1200MPa for 550). The heat treatment described above also increased the ultimate tensile strength of Cell 4 by an estimated additional 10%.

3.3.3 Injection and Flow Through: Cell 5

Oxygen limitation becomes an issue in Cells 1-4, as the sample chamber is sealed while at pressure. Oxygen is essential for the correct folding of fluorescent proteins (such as GFP) at a minimum concentration of 0.1 parts per million (ppm) [155]. It would therefore be desirable to be able to supply fresh media, carrying dissolved oxygen, under pressure. This would also allow the addition of inducers for induction time-course experiments for fluorescent strains. To allow the injection of liquid into the sample chamber, a new approach to the design of the rams, pistons and pressure cells was required (Figure 3.8).

Cells 1-4 have a separate ram piston and pressure cell piston (Figure 3.6). In contrast, in Cell 5 the two pistons were combined. A second, floating, ram was fitted to the modified piston. This creates a holding chamber where the liquid to be injected into the sample chamber can be stored under pressure before injection. The floating ram has a second piston which can be pressurised independently of
the stationary ram, which in turn forces the liquid from the holding chamber into the sample chamber via a one-way valve in the modified piston.

Figure 3.8: Scale drawing and photograph of Cell 5, with injectors/flow through. This system is made up of a pressure cell $\alpha$, a pressure cell piston $\beta$, a stationary ram $\gamma$, a floating ram $\sigma$ and an injector piston $\epsilon$. This system was developed to allow fresh media to be added into the sample chamber potentially mitigating oxygen depletion inside the sample chamber. The diagram is to scale; lengths are given in table 3.3
### Table 3.3: Description and lengths of Cell 5 as illustrated in Figure 3.8.

<table>
<thead>
<tr>
<th>Letter</th>
<th>Description</th>
<th>Distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Overall Width</td>
<td>49</td>
</tr>
<tr>
<td>B</td>
<td>Overall Height</td>
<td>80</td>
</tr>
<tr>
<td>C</td>
<td>Pressure Cell Height</td>
<td>17</td>
</tr>
<tr>
<td>D</td>
<td>Pressure Cell Piston Height</td>
<td>44</td>
</tr>
<tr>
<td>E</td>
<td>Stationary Ram Base</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>Flow Through Port</td>
<td>9</td>
</tr>
<tr>
<td>G</td>
<td>Stationary Ram Body</td>
<td>24</td>
</tr>
<tr>
<td>H</td>
<td>Window Aperture</td>
<td>1.5</td>
</tr>
<tr>
<td>I</td>
<td>Floating Ram Height</td>
<td>30</td>
</tr>
<tr>
<td>J</td>
<td>Floating Ram Piston Height</td>
<td>35</td>
</tr>
<tr>
<td>K</td>
<td>Floating Ram Width</td>
<td>24</td>
</tr>
</tbody>
</table>

The injection of liquid into the sample chamber causes an increase in the volume of liquid inside the sample chamber. If the volume is fixed, this will in turn increase the pressure within the sample chamber. In order to avoid this, a pressure regulator is attached between the hand pump and the stationary ram. The pressure regulator acts like a reservoir, absorbing small changes in pressure as the volume within the sample chamber increases.

The injector system also incorporates a system for the flow-through of liquid at pressure. This allow liquid from an external pressure vessel to be circulated through the microscope pressure cell. The pressure cell and the external pressure vessel can be connected via high pressure tubing and liquid flushed through the pressure cell by moving a magnet along the length of the pressure vessel. A partner magnet is designed to fit within the pressure vessel keeping the “fresh” liquid separate from the “used” liquid returning from the pressure cell.

### 3.4 Pressure Cell Maintenance

The pressure cells are cleaned using acetone, 70% (v/v) ethanol and 10% (v/v) acetic acid. Briefly, after use the pressure cell is opened and washed out with 70% (v/v) ethanol, to kill any bacteria within the sample chamber. A small piece
of lens cleaning tissue is wrapped around the tip of a fine pair of tweezers and
dipped in acetone. This is then used to polish the window free of any debris.
For long term storage, the pressure cell is wrapped neatly in lens cleaning tissue
to prevent scratches to the window. Prior to the next use, acetone is used to
clean the surface of the window, the sample chamber is filled with 70% (v/v)
ethanol and the pressure cell placed in a drying oven to evaporate the liquid
away. Should a visible mark or debris remain in the field of view when imaging a
solution of 10% (v/v) acetic acid can be used as an additional cleaning step prior
to sterilization with 70% (v/v) ethanol. The pressure cells are autoclavable but
in practice the solvent method of cleaning and sterilization has never led to any
detectable contamination.

3.5 Calibration

3.5.1 Pressure Calibration

In order to determine the pressure within the sample chamber it is necessary to
calibrate the pressure gauge on the hydraulic line between the pump and the
ram. Traditionally this is done using the fluorescence spectrum of ruby [156].
The emission spectrum of ruby has two characteristic peaks, known as the R1
and R2 lines. Under pressure the R1 line shifts in wavelength. This shift is
linearly related to the pressure. This calibration method is useful at very high
pressures (such as diamond anvil cells) because it is easy to perform and the ruby
can be left within the sample chamber during the experiment. However for lower
pressures (<100MPa), the shift of the R1 peak is often too small to be measured
accurately. For this reason ruby calibration was used for Cells 1 & 2 but not the
newly developed pressure cells 3, 4 & 5.

Other calibration methods do exist [157, 158, 159] for lower pressures, but
these often use propriety materials, are not as accurate as ruby calibrations or
require fluorescence spectra to be measured at different excitation wavelengths. These methods are therefore unsuitable for calibrating the new pressure cells.

An alternative calibration method, based on the liquid crystal 4’-Pentyl-4-diphenylchlorarsine (5CB) was used. This method relies on the known temperature/pressure dependence of the isotropic-nematic transition for 5CB [160]. As pressure is increased at a known temperature the 5CB will cross a phase boundary causing a shift from a clear liquid to a non-transparent gel. By observing the transition into the isotropic phase at a known temperature, the sample chamber pressure can be measured independently of the ram pressure. The phase transition was detected by shining a polarized laser beam through the pressure cell and hollow ram and increasing the ram pressure until the beam is extinguished. This process can be repeated for several temperatures giving an accurate calibration curve for any pressure gauge. The pressure within the pressure cell corresponding to this ram pressure can be calculated:

\[ T = 40.3P - 2.64P^2 \]  

where \( T \) is the temperature above the ambient pressure transition temperature (in centigrade) and \( P \) is in Kbar (1kbar=100MPa)[160]. One therefore first plots the transition temperature versus ram pressure, then converts this to into a plot of cell pressure versus ram pressure using equation 3.8. This procedure is shown in Figures 3.9 3.10 & 3.11 for Cell 2 and Figures 3.12 & 3.13 for Cell 3.
Figure 3.9: 5CB calibration curve for Cell 2. Phase transition readings taken for increasing pressure (ram up) and decreasing pressure (ram down) for each temperature point. Cell 2 has a maximum pressure of 700MPa. To calibrate to this pressure with 5CB would equate to a temperature of >150° C above room temperature.
Figure 3.10: 5CB calibration curve for Cell 2 after temperature conversion. Using equation (3.8) one can convert transition temperature to sample chamber pressure, this allows ram pressure to be plotted against cell pressure.
Figure 3.11: 5CB calibration curve for Cell 2 superimposed onto ruby calibration and piston ratios for the same cell. The two calibrations methods are in agreement with each other highlighting the validity of the 5CB calibration method against the established Ruby method. This data is also in agreement with the piston ratio calculation.

This calibration method was first tested against the ruby calibration for Cell 2. The results are shown in Figure 3.11. The slight deviation from a perfect match is due to the different gauges used to measure the ram pressure. For the 5CB calibrations a 40 Bar gauge was used whereas for the ruby calibration a 100 Bar gauge was used. At pressures below 1.4MPa the 100 Bar gauge is consistently offset by -0.2MPa, while from 1.4MPa to 4MPa, the two gauges are in alignment.

Results for the calibration of Cell 3 using the 5CB method are shown in Figures 3.12 & 3.13.
Figure 3.12: 5CB calibration curve for Cell 3. Phase transition readings taken for increasing (ram up) and decreasing (ram down) pressure for each temperature point. Cell 2 has a maximum pressure of 70MPa, this is easily calibrated using the 5CB method as it equates to only 27°C above room temperature.
Figure 3.13: 5CB calibration curve for Cell 2 superimposed onto piston ratios for the same cell. The 5CB data is in agreement with the piston ratio calculation therefore proving valid for this pressure cell as well as Cell 2.

### 3.5.2 Optical Calibrations

The optical performance of the pressure cells can be measured by imaging fluorescent beads of a known diameter using a confocal microscope. The 60x ELWD objective (Nikon, Japan) has a correction collar fitted to compensate for varying thicknesses of coverslip, or in this case window. By changing the position of the ring the light path is bent, changing the image quality. Figure 3.14 shows that the optimal position for this ring is at its lowest setting (0.5) for the 60x ELWD objective when used in conjunction with Cell 3.
Figure 3.14: Fluorescence confocal microscopy images of the same 3μm fluorescent bead for different collar settings. From left to right: 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2. Images were captured with a Bio-Rad confocal using a 60x ELWD objective, Scale bars = 3μm.

Optical performance can be assessed by measuring the point spread function for a given optical set up. A 3D object becomes distorted in the z-plane due to imperfections in the imaging set up. As a rule of thumb, an objective with a high NA value will distort the image less than an objective with a smaller NA value. In order to measure the point spread function 3μm beads coated with Fluorescein isothiocyanate (FITC) were imaged in each pressure cell using a Bio-Rad confocal microscope. A series of z-stack images were taken: since the beads are spherical an xz-image with no distortion would be identical to an xy-image. The point spread images for Cells 2-4 can be seen in Figures 3.15 3.16 and 3.17 respectively. These Figures show the amount of distortion in the z-plane is reduced in Cell 3 and 4 with Cell 4 giving the best optical performance.
Figure 3.15: Fluorescent confocal microscopy image of a single fluorescent bead for Cell 2. Left panel: xy cross section, Right panel xz cross section. Images obtained using 20x ELWD objective with ring setting of 0.5 Scale bars = 3µm.

Figure 3.16: Fluorescent confocal microscopy image of a single fluorescent bead for Cell 3. Left panel: xy cross section, Right panel xz cross section. Images obtained using 60x ELWD (NA 0.70) objective with ring setting of 0.5 Scale bars = 3µm.
3.6 Imaging Bacteria at Pressure

The aim of developing the pressure cells was to allow high resolution imaging of bacteria at biologically relevant pressures. It has been shown above that these pressure cells are able to attain the pressures needed, are easy to use and are adaptable to many situations. What follows is a series of images demonstrating that these pressure cells are suitable for imaging bacterial samples while at pressure.

3.6.1 Imaging *P. profundum* at Pressure

In order to image the gene regulation of *P. profundum* in response to osmotic and hydrostatic pressure (my original aim) it was essential that *P. profundum* cells could be tagged with a fluorescent marker. The most obvious fluorescent tag is GFP; this protein has been used in hundreds of research papers as a transcriptional and translational markers in bacteria. In order to tag *P. profundum* with GFP the *gfpmut2* gene was cloned into the arabinose inducible plasmid, pFL190, giving pFL190-GFP. This plasmid was then mated into *P. profundum* SS9 and selected for via resistance to Rif and Strep antibiotics.

Once successful exconjugants were selected and purified they were grown at 0.1MPa and imaged using a Deltavision microscope. As seen in Figure 3.18
the resulting bacteria showed what appeared to be bistable production of GFP regardless of inducer (arabinose) concentration. This was followed up by growing this strain in the plate reader at different inducer concentrations, measuring OD and fluorescence at 15min intervals. These results confirmed that this strain was unaffected by inducer levels (see Figure 3.19 and 3.20).

Figure 3.18: Images of *P. profundum* SS9R pFL190-GFP after induction. A: 0.2% Arabinose, B: 0.4% Arabinose
Figure 3.19: Growth Curves for *P. profundum* SS9R pFL1900-GFP with different levels of inducer. Higher levels of inducer have no effect on the growth of *P. profundum* SS9R carrying pFL190-GFP.
Figure 3.20: Fluorescence chart for *P. profundum* SS9R pFL1900-GFP with different levels of inducer. Higher levels of inducer have no effect on the fluorescence seen in *P. profundum* SS9R carrying pFL190-GFP.

The pFl190 plasmid was specifically designed for use in *P. profundum* [135] and has been used previously to compliment gene deletions in *P. profundum* SS9. This suggests that the levels of GFP expression were being controlled at the translation level and may be due to several factors including codon usage and protein folding [73, 135]. However this is difficult to test and this plasmid construct was abandoned.

Other plasmids were investigated; these included derivatives of pES213, a naturally occurring plasmid found in *Vibrio fischeri* [134]. Different fluorescent proteins were also investigated; these included RFP (both monomers and dimer forms) and LOV-Proteins, in order to identify a suitable tag for *P. profundum*. For a full list of plasmids investigated see Table 2.2.

Of all the plasmid and fluorescent tag combinations investigated none gave
sufficient levels of expression to warrant further investigation. This effectively halted the optical investigation of pressure adaptation in *P. profundum*.

### 3.6.2 *E. coli* Wild-Type Growth at Pressure

*E. coli* is a non-pressure adapted bacteria with rod shaped cells, $\sim 1 \times 2 \mu m$ in size. Its morphological response to pressure has been well documented. Although the details are strain-dependent, strains such as *E. coli* 0157:H7 have a higher pressure tolerance and are better adapted to pressure shocks due to the natural up regulation of the sigma factor RpoS [83, 80].

As a general guide, *E. coli* cells grown at $\sim 25MPa$ are able to grow as rod shaped cells, continuing to divide as normal but are slightly longer than unpressurized cells [161, 162, 163]. At higher pressures, 30-50MPa, cell division is inhibited which causes the cells to become filamented [164, 165, 161, 162, 163]. Growth is abolished at pressures above $\sim 60MPa$ [164, 165, 161].

Pressure cell 3 was used to observe the effect of high pressure on *E. coli*, *in situ*, over several hours. *E. coli* cells were pre-grown in Luria Bertani (LB) media supplemented with 25mM Glucose for 2 hours at 0.1MPa, these cells were then loaded into Cell 3 and allowed to attach to the window surface for 30min. After this time, the media was removed and fresh media added, leaving only the attached cells inside the sample chamber. The pressure cell was then sealed and pressurised to 50MPa and images were taken every 20min. Figure 3.21 shows *E. coli* MG1655 growing at 50MPa at 120min, 620min and 20 hours. At 120min the cells are regular rod shaped but arrested in their growth, while at 620min the bacterial cells show heterogenous filamentation; some cells are filamenting whereas others remain as regular rods. These rods have been observed to be motile. At 20hours the majority of bacterial cells are filamenting however the population remains heterogenous with some cells remaining regular rods. These results are in agreement with previous literature although the population
heterogeneity of filamentation has not been previously commented on. These images demonstrate the suitability of Cell 3 for imaging bacterial cultures while at pressure.

Figure 3.21: *E. coli* MG1655 growing in LB 25mM Glucose at 50MPa in Cell 3. Images taken at 120min (A) showing regular rod shaped cells in which have arrested growth due to change in pressure, 620min showing a heterogenous population where some cells are starting to filament and other remaining regular rods (B) and 20 hours showing a heterogeneous population where both filimented and unfilamented cells can be seen (C). Scale bars = 10µm.

### 3.6.3 *lon* Mutant Filamentation

The SOS response in *E. coli* is induced by damage to DNA. This response halts cell division, allowing time for the damaged DNA to be repaired [165]. This can lead to filamentation where the cell is able to grow but not divide (as seen in Figure 3.21), and can ultimately lead to cell death if division is not able to restart.
It has been shown that a treatment of 200MPa for 15min will induce the SOS response in *E. coli* even though no apparent DNA damage is caused. By deleting the cell division regulatory proteinase Lon hyper-filamentation occurs in outgrowth, producing very long cells which soon die [163]. I therefore hypothesized that filamentation of the *lon* mutant cells at moderate pressure might also be greater than for the wild-type cells.

Figure 3.22 shows the *E. coli* strain BW25113*Δlon* at 0min where the cells are regular rod shapes (A & B) and are able to grow and divide at 0.1MPa until they cover the window entirely (C), however at 50MPa the bacterial cells are hyper-filamented, forming very long rods (D). The observation of hyper-filamentation has, until now, only been observed in cells grown after high pressure treatment (out-growth) [163]. Using Cell 3 I have been able to show that the SOS response is induced in *lon* mutants growing at 50MPa (Figure 3.22). This finding suggests that substrate of Lon (SulA) is being accumulated in these cells preventing cell division.
3.7 Discussion

During my PhD I have, with the help of Hugh Vass, developed a modular optical pressure cell. Several pressure cell designs have been developed by several groups for several different purposes but a common theme runs through all designs: increases in pressure range result in decreases in optical performance. For microbiology increased optical performance is often the greater driving factor in the design of a pressure cell: bacteria are very small and require high magnification optics in order to be seen.
In order to design a pressure cell with optimal optical performance while trying to retain a wide pressure range requires a knowledge of how pressure affects different materials and structures. In the designs for these pressure cells every attempt was made to improve optical performance by using window materials which are better suited to optical microscopy, such as glass and quartz instead of diamond, while adapting the design to retain as much pressure tolerance as possible.

### 3.7.1 Pressure Cell Design

The pressure cell system designed during my PhD is made up of a ram and ram piston module, four individual pressure cells, each with different optical and pressure limitations, and pressure cell pistons. The ram and pressure cell piston can be either of a hollow or solid design, allowing for transmitted light microscopy and very high pressure work respectively. Due to the modular nature of this design it is possible and easy to produce other pressure cells with different window designs allowing for greater flexibility.

There are, however, several limitations to the pressure cells described here. The first is the optical quality of the original two pressure cells: the optical resolution is too low to investigate the physiology of bacterial cells at high pressure. It was for this reason that I embarked on the development of the third pressure cell. This pressure cell, cell 3, was designed to fit a Nikon Bio-Rad confocal microscope with a 60x Extra Long Working Distance (ELWD) objective with a maximum working distance of 2mm and an NA of 0.70. Due to the increase in optical performance there was a trade off with the maximum pressure obtainable, which was reduced significantly from 700MPa to 100MPa compared to cell 2.

A fourth pressure cell was also designed with further increased optical performance. This required a total rethink of the way in which the window
is supported. In the previous pressure cell designs the window is supported by a metal surround, which is part of the pressure cell body. Using a poulter type seal these supports are very effective at withstanding pressure and are very common in pressure cell designs. In the fourth pressure cell this support has been removed entirely thanks to a revolutionary new window design. This fourth pressure cell was designed to fit a 60x Wi objective with a working distance of 0.27mm and an NA of 1.20, giving it a significant optical advantage over pressure cell 3.

Further to the physical trade off between maximum pressure and optical resolution there are biological limitations to take into account in the design of pressure cells. The primary biological limitation is the oxygen levels within the pressure cells after the application of pressure. In the absence of oxygen, GFP and similar fluorescent proteins do not fold correctly and thus do not fluoresce. The large (∼100µl) sample volume of the pressure cells means that some oxygen is available inside the cell but after ∼6 hours this oxygen has been used for aerobic metabolism as shown by the loss of GFP fluorescence.

In order to overcome the limitation of oxygen availability I also developed another pressure cell system, which allowed injection into the sample chamber. This fifth design also allows the flow of liquid from a pressure vessel into the sample chamber for optical examination. However by having the injection ram floating above the pressure cell ram the ability to view samples with transmitted light was lost. For fluorescent microscopy the lack of transmitted light is not an issue but for cell physiology work, phase contrast is often desirable making this system less useful than the previous pressure cells.

This fifth pressure cell was not fully completed because materials for attaching and detaching the external pressure vessel were difficult to obtain or build. However the injector worked as designed and it is also possible to remove sample from the pressure cell via the injection assembly.

One of the driving factors for developing these pressure cells was to create a system which has as many potential applications as possible, and with the
modular design this is indeed the case. The two original pressure cells are capable of pressurising bacterial samples to pressures up to 700MPa, so that killing can be observed. If this is combined with confocal microscopy it may be possible to observe some of the physiological effects of pressure on bacterial cells. This type of study would go a long way to addressing some of the questions raised by depressurization of samples for observation. It is still unclear if the physiological effects seen in samples treated at lethal pressures are due to pressurisation or depressurization.

If the flow through system of pressure cell four could be build into the standard ram of the other pressure cells it would open up the possibility of observing biofilm formation at pressure, something which, to my knowledge, has never been attempted. The regular addition of oxygenated liquid would counter the problems of poor fluorescence and the “flow-cell” design would be within keeping of other biofilm experiments. In these experiments flow-cells are used to remove non-bound bacteria ensuring that only the biofilm remains in the sample chamber.

It was shown by the Bartlett lab in 2008 that *P. profundum* utilizes different flagella at different pressures [166]. In this study each cell was observed, tracked and its swimming speed calculated. This process is rather laborious. Another way of measuring swimming speed is to use Differential Dynamic Microscopy, DDM [167], which uses out of focus transmitted light under lower magnifications (typically 10-20x) to record high speed video data which can be used to measure swimming speed of bacteria. DDM is entirely compatible with pressure cells 1-3 as they allow for transmitted light microscopy and this technique is now starting to be used in the School of Physics with pressure cell 2.

The fourth pressure cell with its much better resolution can be used to track single molecules of GFP at high pressure, something which has not been attempted before. With the use of high NA objectives techniques like TIRF (Total Internal Reflection Fluorescence) microscopy become available allowing very exciting new experiments to be carried out. Initial steps in this direction
are now being taken in collaboration with Cristina Flors (School of Chemistry). Furthermore we are exploring the possibility for commercial (Linkam Scientific) as well as academic (Catherine Royer, Montpellier, France and Abram Aertsen, Leuven, Belgium) collaborations. Within the University of Edinburgh, a new student, Diarmuid Lloyd, is already developing this pressure cell further.

3.7.2 Pressure Cell Calibration

Another development in this project was the use of the liquid crystal 5CB (4’-Pentyl-4-diphenylchlorarsine) as a method of calibrating pressure within the pressure cells. In order to be effective this calibration needed to be equal to or better than ruby fluorescence calibrations. I believe the use of 5CB is superior to ruby fluorescence in specific applications. Ruby fluorescence is ideal for work at very high pressures such as that done with diamond anvil cells however, the shift in the spectrum is so small at lower pressures that this method is very difficult to use. The 5CB calibration on the other hand is simple and does not require any specialist equipment. Furthermore the 5CB calibration method can be done for any pressure gauge, no matter how inaccurate, as it relies on temperature to identify the pressure phase transition. This reduces the overall cost of the pressure cells as accurate pressure gauges can be a significant investment.

The one major limitation the 5CB calibration method has compared to ruby fluorescence is that in situ calibration is not possible. By this I mean that ruby can be glued in place during an experiment and its fluorescent spectrum measured through out, giving realtime pressure readings. This cannot be done with 5CB as it would contaminate the sample.

3.7.3 Image quality

Image quality is of great importance when developing equipment and techniques for microscopy. In order to test image quality I measured the point spread
functions for several pressure cells. The point spread function gives a good estimation of the amount of distortion caused by the window thickness as well as the window material. For instance a thick diamond will have greater distortion than a thin glass coverslip. This is because of the very high refractive index of diamond does not match up with the refractive index of the objective lens.

Comparing the point spread functions for cells 2, 3 and 4 it became apparent that the change from diamond (cell 2) to quartz (Cells 3 & 4) as a window material made a vast difference in the image quality. Part of this is be due to the change in objective used for these experiments, the 20x ELWD objective has an NA of 0.45 where as the 60x ELWD has an NA of 0.70. Further improvements can be made to image quality by adjusting the correction collar of the objective, this is possible with the 20x and 60x ELWD objectives but not with the 60x Wi objective.

The fact that these pressure cells have been designed for use with “standard” objectives provides significant advantages when adapting to other microscope systems. In the case of [118] a specially constructed objective was used to gain better magnification and NA values for use with a diamond anvil cell. This diamond anvil cell can only be used on one microscope making it less adaptable, whereas the pressure cells described here can be transferred to other makes and models of microscope. In fact this has happened several times during this project. The original pressure cells were designed to be used with a Nikon confocal microscope. Later the addition of a small adapter allowed the pressure cells to be used on a Nikon epi-fluorescence microscope, and recently a new adapter has been made allowing these pressure cell to be used on a Carl Zeiss confocal microscope.

3.7.4 Future work and Prospects

In this section I will discuss the possible future developments of the pressure cells, their possible applications and work being started by new students in the lab.
Since publishing the designs of the pressure cells [168] several labs have shown interest in obtaining and/or using this equipment. During a visit by Abram Aertsen to our lab an important flaw in the design was noted: our system is approximately 5mm too tall to allow for phase contrast microscopy. This problem is further compounded by the very small aperture in the hollow piston which does not allow the full cone of light emitted from the phase contrast condenser to be focused on the sample within the pressure cell. The images in this thesis use transmitted light, not phase contrast microscopy. In order to overcome this limitation a design incorporating two side pistons, instead of a single top piston, is currently being developed. This would mean a larger, thicker window could be placed on the upper surface of the sample chamber allowing the whole cone of light from the condenser to enter the sample chamber unobstructed. This design would also have a smaller height as the ram would no longer be on the top of the pressure cell but at its sides.

Further concerns with the current design include the weight of the system. More advanced microscopes have motorized stages allowing the tracking of XY coordinates during an experiment; however these stages have a maximum weight limit of less than 1kg. The total weight of the current pressure cell system, including heating elements and stage attachments is in excess of 900g, making it borderline as to whether it can be used with these microscopes. Ways to reduce weight are being investigated, including the incorporation of the heating elements, stage attachments and rams into one single device. This would reduce the weight by over 100g and have no effect on the pressure range; however the building of such a complex part would need different manufacturing techniques, such as “lost wax casting” which require very skilled labor and specialist equipment which are not available to us at this time. Another way of reducing weight is to minimize the size of the pressure hose used from the hand pump to the pressure cell. Currently this is 1/8 inch hose. If this was reduced to 1/16 inch tubing, as used for HPLC, then there would be a weight reduction of roughly 25-50g.
Possible new experiments include the use of cell 4 to investigate the effects of pressure at a single molecule level. Using TIRF microscopy it is possible to track single molecules of GFP with high temporal resolution. In TIRF the evanescent wave within the first 50-200nm of the coverslip’s internal surface excites fluorescent molecules making them detectable without significantly increasing photo-bleaching. The limit of 50-200nm of the coverslip’s internal surface means that TIRF is best used with membrane bound proteins tagged with GFP and this suggests a very exciting experiment: the measurement of membrane bound protein movement through the membrane pressure/temperature phase boundary. This can be done in bacterial cells; initial experiments have shown SerA to be a good candidate to be tagged with GFP and its movement traced. The expected result of this experiment is that as the pressure causes the membrane to change from a liquid crystal to a gel the speed and/or amount of movement should be drastically reduced. By using high speed cameras and particle tracking software it should be possible to get statistically robust data for this change in movement within the membrane as a result of pressure.
Chapter 4

Effects of Hydrostatic and Osmotic Pressure on Wild-Type Strains of *P. profundum*

Understanding the growth of bacteria under stressed and unstressed conditions allows one to gain insights into how a specific stress effects that bacteria. In order to gain proper understanding it is useful to investigate the whole rage a stress rather than taking “snap-shots” at selected points within that stress. Here I am using both hydrostatic and osmotic pressure as stresses in combinations. To avoid focusing on snap-shots I needed to measure growth at a large number of osmotic and hydrostatic pressures: this meant a large number of cultures were needed to get this high resolution data. Without this high resolution data it is possible that the subtle effects of this combination may have been missed.
4.1 Developing a quantitative method for high-throughput growth at elevated pressure

The standard method of growing bacteria at pressure is to use a flexible, sealable container within a pressure vessel. This container is normally a plastic bag or a bulb made from the end of a pipet as these are easily sealed and very flexible. Less often the culture is grown directly in the pressure vessel, however this means that only one culture can be grown at a time and specialist pressure vessels must be used so that contamination is eliminated.

There are several limitations to using bags and bulbs for growth of bacteria at pressure. Bulbs cannot be sampled from and resealed, making it impossible to measure one sample over several time points. Traditionally, for each time point a bulb is sacrificed from a set of multiple bulbs set up from the same starter culture. This method introduces increased variation into the data as the bacteria may not grow in exactly the same manner in each bulb, which in turn makes meaningful analysis more difficult. Bags have different problems: they are large enough to allow multiple samples to be taken and the bag re-sealed each time, however taking samples requires the use of needles and syringes to pierce the bag and draw a sample. The outer surface of the bag is not sterile and in fact likely to have a multitude of bacteria attached from the pressurisation media used in the pressure vessel. In piercing the bag some of these bacteria may be able to enter the bag causing contamination. To overcome this problem ethanol can be used to clean the area being sampled from; this is effective, but it substantially increases the time the bag is out of the pressure vessel allowing the samples to start to adapt to 0.1MPa, altering the quality of the data obtained. The use of ethanol as a pressurising medium can be discounted as this would remove sample labels and poses a possible health and safety problem.

To overcome these issues I decided to investigate other methods for growing bacteria at high pressure. The first attempt was to use “blood bags”; these are
bags used to store blood after donation and have the advantage of a self-sealing port from which samples could be taken quickly. These ports are also small enough that they could be cleaned quickly, reducing the time the samples are out of the pressure vessel. Initial tests showed cultures grown in these bags had the same growth rates as using other bags. However the cost per bag was prohibitive; each bag costs £15. An alternative, less expensive, method was therefore needed.

Finding a way to seal a 96-well plate so as to allow it to be pressurised in the 3 liter pressure vessel would allow the same sample to be measured multiple times without destroying the sample. In addition, because the plate reader can measure 96 samples very rapidly the time that each sample is kept out of the pressure vessel would be kept to a minimum.

To seal a 96-well plate in this way was not however a simple matter for several reasons: each well has to be kept separate from its neighboring wells to avoid cross-contamination, the plate as a whole has to be kept separate from the pressurising medium, the light path from the top of the sample to the bottom of the sample must be kept clear in order to measure OD$_{(600nm)}$ accurately, and the seal must be flexible enough to transmit pressure into the samples.

Firstly attempts to use standard clear 96-well plate films were made. This resulted in the pressurising medium entering the gap between the plate and seal, causing the film to lift off and the samples to be lost in the pressurising medium, as shown in Figure 4.1. To avoid this, these films were then used in conjunction with a plastic bag; however it was not possible to remove enough air from the bag to stop the bag from breaking when pressurised, upon which the pressurising medium would enter the bag and the film would lift off releasing the samples into the pressurising medium.

Heat-adhered films were also tried. These films require a heat source to be used to bond the seal to the plate. Throughout the initial tests these films remained firmly attached to the plate while at pressure; however these films are made of silver foil making it impossible to measure OD$_{(600nm)}$ in the plate reader.
Figure 4.1: Photograph of a 96-well plate sealed with a standard transparent film. The plate was filled with a crystal violet stain allowing for easy identification of leaked wells. In the bottom left corner of the plate it can be seen that the film has separated from the plate.

I also tried out clear heat-adhered films, but these would not bond to the plate under any conditions, so were not tried in the pressure vessel.

In an attempt to strengthen the bond between normal 96-well plate films and the plate a thin layer of epoxy resin was dotted between each well and the film placed on top as shown in Figure 4.2. A further beading of epoxy was placed around the edge of the plate, ensuring the pressurising medium was kept out. This method did keep each well separate, transmit pressure and, for the majority of wells, allow $\text{OD}_{(600\text{nm})}$ to be measured. For some wells, the additional epoxy had spilt over into the light path making it difficult to obtain accurate $\text{OD}_{(600\text{nm})}$ measurements. This was deemed to be due to a lack of practice in applying the epoxy to the plate so it was decided the method should be developed further.

After several attempts at using these modified plates it became apparent that the overflow of epoxy into the wells was related to the volume of sample in each well, so the volume was reduced. This introduced another problem: too much trapped air would cause the film to break upon pressurisation and small bubbles would affect the $\text{OD}_{(600\text{nm})}$ measurements.
Figure 4.2: Schematic diagram of a 96-well plate sealed with epoxy resin. Epoxy resin was dotted in the gaps between wells and a clear film placed on top of the plate creating a seal. Small amounts of epoxy resin would enter the wells contaminating the contents or trapping air bubbles making OD measurement difficult.

While trying to overcome this issue I, by accident, ran out of films, so borrowed several of a different brand from the Gene Pool sequencing service. These MicroAmp films had a thicker layer of adhesive across the surface, so I tried these new films without the epoxy dotted between the wells, but maintaining the epoxy around the edge of the plate. These plates performed as well as the epoxy dotted plates but had fewer, if any, air bubbles, thus the OD$_{(600\text{nm})}$ measurement were more accurate.

Figure 4.3: Schematic diagram of a 96-well plate sealed using MicroAmp films with an epoxy surround. By using the MicroAmp films it was possible to only use epoxy resin on the outer edge of the plate, removing any chance of epoxy contaminating the samples and reducing the size and number of air bubbles.

The major drawback to this method of sealing the 96-well plates was that the thicker adhesive on the film would become opaque over time. This clouding is probability due to the adhesive reacting with the sample over time, causing the
adhesive layer to become more opaque. Fortunately this gradual clouding was measurable therefore it was possible to eliminate it as a variable (See Figure 4.4). In order to eliminate the clouding effect from sample data all that is required is a blank well containing media alone (i.e. no bacteria). Any changes in OD\(_{(600nm)}\) of this well are due to clouding of the film and can be subtracted from the data for the remaining 95-wells. Tests were carried out to evaluate the toxicity of the adhesive and no observable effects were seen on growth of bacteria in plates sealed with MicroAmp films as compared to growth in sealed bags.

![Figure 4.4: Measurement of 96-well plate seal clouding over time. Red: Empty wells containing only MOPS minimal media. Blue: Wells containing MOPS minimal media and \textit{P. profundum} SS9. OD was measured for 18hours at 0.1MPa, 15\(^\circ\)C with shaking at 200rpm. OD\(_{(600nm)}\) for empty wells increases as the seal adhesive becomes water logged becoming saturated after approximately 1 hour. OD\(_{(600nm)}\) for cultures is variable with maximum OD\(_{(600nm)}\) ranging from 0.5 to 1.0](image-url)

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Having thus developed and tested a method of sealing 96-well plates I then set about using these plates to measure the growth rates of different strains of *Photobacterium profundum* at a range of pressures (from 0.1MPa to 50MPa) and at a range of NaCl concentrations (from 100mM to 700mM). The results of these experiments are presented in the following sections.

### 4.2 Growth of *P. profundum* SS9 and 3TCK as a Function of Pressure and Salt Concentration

Using the newly developed high-throughput method for growth at elevated pressure I measured the growth rates of two different strains of *P. profundum* at 4 pressures and 11 NaCl concentrations. I chose to use MOPS Minimal Media (MMM) as a growth medium for these experiments as it had previously been used with *P. profundum* in carbon starvation experiments [98, 169] and it would allow for full control over the NaCl concentration, as well as any other solutes which were later chosen as variables. The main carbon source for these experiments was glucose, at a concentration of 25mM, with 0.1% casamino acid supplement. The casamino acids were not sufficient to act as a carbon source, but were sufficient to allow growth - *P. profundum* would not grow in MMM without this supplement.

Throughout all experiments with *P. profundum* the temperature was maintained at 15°C. This is the optimal growth temperature for *P. profundum* SS9 as shown by DeLong [4].

For each strain and each pressure, starter cultures were set up at 0.1MPa in Marine Broth. These starter cultures were diluted 1:100 into MMM (400mM NaCl) and incubated statically at 0.1MPa for 48 hours. A 1:100 dilution of the MOPS cultures were made into fresh MOPS with 11 different concentrations of NaCl (100-700mM) and aliquoted into the 96-well plate in replicates of 4 or 8. In
the 12th column on the plate dilutions of crystal violet at known absorbance at 600nm ranging from $A_{(600\text{nm})}$ 0 to 1.3 were added, in order to monitor the change in $\text{OD}_{(600\text{nm})}$ in wells not containing bacteria due to clouding of the MicroAmp films. These wells also acted as internal controls ensuring the blank subtraction method was accurate over a range of $A_{(600\text{nm})}$ figures.

The plates were then sealed, their $\text{OD}_{(600\text{nm})}$ measured in the plate reader (time = 0) and pressurised. $\text{OD}_{(600\text{nm})}$ was measured after 12hours and then again at 3hour time intervals for up to 80 hours in total. By logging ($\text{LOG}_{10}$) the $\text{OD}_{(600\text{nm})}$ data, manually measuring the gradient of the exponential growth phase and multiplying by 2.303 the growth rate for each sample was calculated. The mean and standard deviation were also calculated for replicates of the same experiment. This was repeated for up to 3 plates for each pressure. For each strain, the growth rate could be plotted in a “growth map” was plotted, showing the growth rate as a function of pressure and salt concentration. One can also make a similar plot of growth yield as a function of pressure and salt concentration.

### 4.2.1 Growth of *P. profundum* SS9

The growth map for *P. profundum* SS9 turned out to be more complex than expected; showing three distinct peaks (see Figure 4.5). It was expected that growth rate would show a single peak at the optimal pressure (this is known to be 28MPa in Marine Broth). A single optimum for NaCl concentration was also expected, somewhere between 250mM and 500mM NaCl, these being the NaCl concentrations used by the Bartlett group in conjunction with Marine Broth.
Figure 4.5: Growth rate map of *P. profundum* SS9 at a range of pressures and NaCl Concentrations. There are three different peaks in the growth rate of SS9, low NaCl and high pressure, high NaCl and high pressure and moderate NaCl and moderate pressure, suggesting a complex relationship between adaptation to NaCl and pressure.

Because the three peaks for the *P. profundum* SS9 growth map are so unexpected it is important to also present the same growth rate data as a function of salt concentration only. This allows the standard deviation of the mean to be plotted also (as error bars), showing the levels of error within the data. As shown in Figure 4.6 the error bars are much smaller than the observed changes in the growth rate, this suggests the observation of three distinct growth optima is robust.
Figure 4.6: Growth rate data for *P. profundum* SS9 as a function of pressure and salt concentration. By plotting growth rate data as a function of salt concentration only is it possible to plot error bars on the same chart: these show that the error within the samples is very small, only increasing slightly at 50MPa. This confirms that the three peak growth map for *P. profundum* SS9 is accurate.

The first of the three observed peaks in growth rate is at 200mM NaCl and 50MPa, the second peak is at 300mM NaCl and 10MPa and the third peak is at 500mM and 50MPa. Interestingly none of these optima peaks coincides with the known pressure optima for *P. profundum* SS9 in Marine Broth. This may be due to the very different nature of MOPS Minimal Media and Marine Broth: MMM is a minimal medium, forcing the bacterium to synthesis the majority of its cell components *de novo*, while Marine Broth supplies a wide range of “starter molecules” which the bacterium does not need to synthesize *de novo*.

Comparing these results to the growth yield maps for these same experiments
is very intriguing. In the growth yield maps the topography of the pressure/NaCl map becomes an “island” having a single central peak with little or no growth at the extremes of either pressure or NaCl (see Figure 4.7). The only exception to this is at 50MPa and 350mM NaCl. At this intermediate salt concentration there appears to be a synergy between NaCl and pressure which allows for improved yield but not growth rate.
Figure 4.7: Growth yield map of *P. profundum* SS9 at a range of pressures and NaCl concentrations. Unlike with growth rate, the growth yield forms an "island" pattern whereby yield is reduced at extremes of pressure and NaCl. The large plateau in this center shows that *P. profundum* SS9 is able to grow to a high density over a wide range of pressure and salt conditions. The same data is also plotted as a set of bar charts for ease of reading and also to show the error within the measurement.
The growth rates of *P. profundum* 3TCK at the same pressures and NaCl concentrations were also measured. This allows for a direct comparison of the two strains and their ability to cope with different osmotic and hydrostatic pressures. This is an interesting comparison because the two strains appear to have very distinct growth patterns in regard to pressure (SS9 is piezophilic where as 3TCK is not) and it is possible that *P. profundum* 3TCK is better able to adapt to changes in salinity due to its isolation in the San Diego bay. My results show that *P. profundum* 3TCK has shows increased growth rates at lower pressures and over a wide range of NaCl concentrations (100mM to 600mM). As pressure increases from 0.1MPa to 10MPa the growth rate at high and low NaCl starts to reduce. By 30MPa strain 3TCK is growing best at 300-400mM NaCl and by 50MPa all growth has stopped. This pattern is shown clearly in Figure 4.8. This plot also shows an interesting interplay between salt and pressure tolerance: at higher pressures the range of salt tolerance is reduced.
Figure 4.8: Growth rate map of *P. profundum* 3TCK at a range of pressures and NaCl concentrations. The growth rate maximum for *P. profundum* 3TCK is below 30MPa and between 150mM and 500mM NaCl, outside of these growth rate is reduced until 50MPa where no growth is observed. The same data is also represented below in four separate charts with error bars. In these charts the effect of pressure can be seen by the clipping of the salt tolerance of *P. profundum* 3TCK at 30MPa and the lack of growth at 50MPa. The same data is also plotted as a set of bar charts for ease of reading and also to show the error within the measurement.
In contrast to *P. profundum* SS9 the growth rate and growth yield maps for *P. profundum* 3TCK are almost identical. At lower pressures and from 100-600mM NaCl, strain 3TCK is able to reach a high cell density which is reduced as pressure increases to 30MPa, at which point high cell yields are seen at 300-400mM NaCl only. These data are illustrated in Figure 4.9.
Figure 4.9: Growth yield map of *P. profundum* 3TCK at a range of pressures and NaCl Concentrations. Maximum growth yield is observed below 30MPa and at salt concentrations from 150mM to 500mM. This data qualitatively matches the growth rate data for this strain. The same data is also plotted as a set of bar charts for ease of reading and also to show the error within the measurement.
4.2.2 Effects of External Solutes of Growth of *P. profundum*

It is well known that in response to increases in external solute concentration bacteria will take up or synthesize compatible solutes in order to balance osmotic pressure across the cell membrane. In response to increased pressure and salt concentrations *P. profundum* SS9 accumulates large amounts of betaine, glutamate, β-HB and alanine [53].

The data above shows only the effects of *de novo* synthesis of compatible solutes, since the MOPS minimal medium does not contain any betaine or β-HB. The levels of amino acids supplied are only sufficient for cellular growth and not high enough to be accumulated within the cell in order to counter the effects of high salt concentration. In order to differentiate between the synthesis and uptake of compatible solutes I decided to measure growth yield for cultures of *P. profundum* SS9 and 3TCK in MOPS minimal medium supplemented with two different compatible solutes.

The chemical β-hydroxybutyrate is very similar to the recreational drug γ-hydroxybutyrate and as such it is a controlled substance in the UK. This makes it both expensive and difficult to obtain; there are numerous forms and police checks involved. I was able to obtain 0.5g of β-HB, a sufficient amount to use as a standard for HPLC analysis but not enough to supplement growth media. For this reason I chose to use Trimethylamine N-oxide (TMAO) as a supplement. TMAO has been shown to be accumulated in deep sea organisms and may have a play a role in piezoprotection [51, 52]. Betaine was also used as it is accumulated by *P. profundum* SS9 in response to changes in salt concentration.

Using the same methods as described above I measured the growth yield for *P. profundum* SS9 and 3TCK at 0.1MPa, 30MPa and 50MPa at salt concentrations from 100mM NaCl to 700mM NaCl in MOPS minimal medium supplemented with either 4mM TMAO or 4mM betaine. The growth yield maps for these
experiments can be seen in Figure 4.10 and 4.11 for \textit{P. profundum} SS9 and 4.12 4.13 for \textit{P. profundum} 3TCK.
Figure 4.10: Growth yield map of *P. profundum* SS9 in media supplemented with 4mM betaine. As pressure increases the growth yield of *P. profundum* SS9, in the presence of 4mM betaine increases at moderate salt concentrations and higher salt concentrations but growth yield is reduced at lower salt concentrations. Interestingly the growth yield at 0.1MPa appears to be slightly higher than at 10MPa.
Figure 4.11: Growth yield map of *P. profundum* SS9 in media supplemented with 4mM TMAO. As with the addition of betaine, the growth yield maps of *P. profundum* SS9 in the presence of TMAO shows an increase in growth yield at moderate and high salt concentrations as pressure is increased.
Supplementing the growth medium of *P. profundum* SS9 with either betaine or TMAO has the effect of increasing growth yield at higher salt concentrations and higher pressures. In the absence of these solutes growth yield is greatest at 30MPa between 250mM and 500mM NaCl. When supplemented with 4mM betaine maximum growth yield is at 50MPa between 250mM and 600mM NaCl. The addition of TMAO to the growth media results in a maximum growth yield between 30MPa and 50MPa and 250mM and 700mM. As the effect of both these compatible solutes is very similar this suggests it is not an effect of specific to either betaine or TMAO which is causing the increased tolerance to both pressure and salt concentration but a more general effect of supplying compatible solutes.

There are some differences in the effects of betaine and TMAO on growth yield which can be attributed to specific actions of these compatible solutes. The fact that betaine shows greater improvement of growth yield at higher pressure suggests that *P. profundum* is physiologically better suited to this compatible solute than to TMAO. Another possibility is that *P. profundum* SS9 is not able to import TMAO into the cell so that the results seen are due to passive diffusion across the membrane.
Figure 4.12: Growth yield map of *P. profundum* 3TCK in media supplemented with 4mM betaine. As pressure increases the growth yield of *P. profundum* 3TCK becomes reduced at lower salt concentrations. This is a similar pattern as when this strain is not grown in the presence of 4mM betaine. The overall growth yield for *P. profundum* 3TCK supplemented with 4mM betaine appears to be lower than that of the unimplemented strain, this could indicate a negative effect of betaine on growth of this strain. The growth map appears to be “creating” data in its generation, this is clearly seen at 10MPa and 700mM NaCl, in the bar charts this data point is somewhat similar to the 600mM data point, however on the growth map plot this is set to 0 growth. I have no explanation for this other than these charts were made in Microsoft Excel.
Figure 4.13: Growth yield map of *P. profundum* 3TCK in media supplemented with 4mM TMAO. This is a similar pattern as when this strain is not grown in the presence of 4mM betaine. The growth map appears to be “creating” data in its generation, this is clearly seen at 10MPa and 700mM NaCl, in the bar charts this data point is somewhat similar to the 600mM data point, however on the growth map plot this is set to 0 growth. I have no explanation for this other than these charts were made in Microsoft Excel.
The addition of betaine also increases both the pressure and salt optima for *P. profundum* 3TCK. However, the addition of betaine appears to have a negative effect on the growth yield of *P. profundum* 3TCK compared to when it is grown without betaine in the growth medium. For growth without supplemented betaine the growth yield is, for the most part, above OD(600nm)1.0 but when grown with 4mM betaine the growth yield only goes above OD(600nm)1.0 at 500mM and between 0.1MPa and 30MPa. This is an interesting paradox: the increase in pressure and salt tolerance suggests betaine is beneficial to *P. profundum* 3TCK but the reduced growth yield overall suggests it is deleterious. With the addition of TMAO to the culture medium the growth yield of *P. profundum* 3TCK is increased only at high salt concentrations, it has no effect on the pressure tolerance of this strain. This, in combination with the results for the addition of TMAO to *P. profundum* SS9, suggests that the observed pressure tolerance effects of TMAO *in vitro* are not applicable *in vivo* with *Photobacterium* strains.

It is important to point out that the effects seen for both *P. profundum* strains with both betaine and TMAO are a shift in the pressure and salt optima, not an extension of the pressure or salt ranges.

I have used growth maps throughout this thesis as a way of representing growth rate and growth yield as a topological map, highlighting the areas for which high growth is observed in combinations of pressure and salt concentration. Unfortunately these maps are not ideal to read and they “create” data between data points. For this reason I have also included with every growth map a set of bar charts of the same data. While not able to show combinations of salt and pressure as easily these charts are able to show error within the measurement and do not plot made-up data.
4.3 The role of ToxR protein in adaption to hydrostatic and osmotic pressure in P. profundum

The ToxR protein is a global regulator of gene expression in most Vibrionaceae and has been best studied in Vibrio cholerae because of its importance in the regulation of cholera toxin, the causative agent of cholera. ToxR is a membrane bound protein with OmpR-like membrane spanning regions. It acts as a “one component” system, meaning it is able to sense both environmental changes and to regulate gene expression directly by binding to DNA. The ToxR protein can form a dimer with itself or a trimer with ToxS (toxR and toxS form an operon) and it is believed that in this trimeric configuration the ToxR/S membrane complex is able to sense changes in pressure [103]. As pressure increases, the fluidity of the membrane is reduced and it is believed that this causes the ToxR/S trimer to dissociate, which allows the ToxR dimer to act alone and regulate its target genes. As of yet it is not fully known which genes ToxR regulates in P. profundum, but in V. cholerae it is known to regulate 154 genes (60 up-regulated and 94 down-regulated) the largest group of which function as transport proteins (22.7%) with the next largest functional group being metabolism (20.1%) followed by pathogenicity (14.3%) [170].

Interestingly the only microarray data on P. profundum SS9 suggests that both transport and metabolism genes are strongly affected by pressure [108]. Since it is presumed that P. profundum senses pressure changes through ToxR/S [103] and that pressure has similar effects on P. profundum as a toxR deletion in V. cholerae (i.e. the altered regulation of outer membrane proteins) it seems logical to suppose that ToxR plays a key role in pressure adaption in P. profundum SS9. For these reasons I chose to study the effects of pressure and salt on a toxR mutant strain, TW30, kindly supplied by Doug Bartlett [98].
The TW30 strain has a frame shift deletion in the toxR gene resulting in the loss of expression of both toxR and toxS. This strain has a high pressure phenotype, the continuous expression of OmpH and no expression of OmpL regardless of growth pressure.

4.4 Sequence analysis of the toxR gene from *Vibrionaceae* species

The toxR gene is 848bp in length, and encodes a 292aa protein. Using p-distance models I constructed a phylogenetic tree based on the sequences of all available unique toxR genes. These results are shown in Figure 4.14.
Figure 4.14: Phylogenetic tree of toxR genes in 18 Vibrionaceae species constructed using a p-distance model. *P. profundum*, at the top of the tree, has little or no sequence difference to other Vibrionaceae toxR genes, suggesting that pressure adaption was not needed for ToxR to sense changes in pressure.

As shown in Figure 4.14, *P. profundum* forms a small cluster at the top of the tree. However the genetic distance between the *P. profundum* sequences and the other Vibrionaceae species is not significantly greater than those found amongst the Vibrionaceae strains. These findings suggest that the structure of ToxR in *P. profundum* is not adapted to sensing pressure and therefore it is likely that the ToxR protein’s already existing response to pressure in other Vibrionaceae species has been hijacked by *P. profundum*. In order to test this one could try and cross-compliment different Vibrionaceae toxR genes into the TW30 strain and test for the restoration of wild-type phenotype.
4.5 Growth data for *P. profundum* TW30

The growth of TW30, the *toxRS* mutant strain of *P. profundum*, was also measured using the high-throughput growth method. The resulting growth rate and growth yield data can be seen in Figures 4.15 and 4.16.
Figure 4.15: Growth rate map of *P. profundum* TW30 at a range of pressures and NaCl concentrations. No growth is seen below 200mM NaCl or above 600mM NaCl with a growth rate maxima at 10MPa between 300mM and 500mM NaCl. The same data is also represented below in four separate charts with error bars. These charts show an increase in growth rate as pressure increases to 10MPa, decreasing at 30MPa with a reduction in salt tolerance at 50MPa. The same data is also plotted as a set of bar charts for ease of reading and also to show the error within the measurement.
Compared to \textit{P. profundum} SS9 the growth rate map of TW30 forms a very simple pattern with little or no growth below 200mM NaCl or above 600mM NaCl. The pressure range of this strain does not appear to be affected by the deletion of \textit{toxRS}. The absence of the three peaks seen in the growth rate of \textit{P. profundum} SS9 suggests that ToxR does play a role adaption to pressure and salt. This is further backed up by the absence of growth at lower salt concentrations; it appears that ToxR is required to maintain osmoregulation at low salt concentrations.
Figure 4.16: Growth yield map of *P. profundum* TW30 at a range of pressures and NaCl concentrations. The same data is also plotted as a set of bar charts for ease of reading and also to show the error within the measurement.

The growth yield data for *P. profundum* TW30 shows an “X” pattern with increased salt tolerance at high and low pressure but a reduced salt tolerance at moderate pressure. Why this is the case is not clear, but I suggest that there is a
synergistic effect of pressure and salt whereby the stressful pressures of 0.1MPa and 50MPa help protect the cells against salt stresses. The fact that this pattern is not seen in the growth rate data is also a mystery, however the growth rate maximum does coincided with one arm of the “X” at 10MPa and 500mM. There is also an extension of the growth rate at 350mM and 50MPa which coincides with another arm of the “X” in the growth yield data.

4.6 Complementation of TW30

In order to confirm the link between the TW30 strain’s genotype and the observed growth phenotype it was important to compliment the mutation in the toxR gene by supplying this gene on a plasmid. The TW30 strain has a frame-shift deletion mutation (a deletion causing a frame-shift) which results in lack of expression of the toxS gene as well as the toxR gene. Therefore in order to gain further insight into the role of ToxR in pressure adaptation I decided to introduce toxR and toxS separately into the TW30 background.

I decided to use the conjugation vector pFL190 which was produced by the Bartlett lab [135], as this plasmid has been shown to have high rates of transfer into P. profundum [135] and has been used by previous lab members successfully. The toxR and toxS genes were successfully cloned by PCR from P. profundum SS9, cut with SpeI and EcoRI and inserted into pFL90 yielding plasmids pFL190-toxR and pFL190-toxS. The inserts were confirmed by PCR using primers for pFL190 and resulted in fragments of the expected size as shown in Figure 4.17. These plasmids were then transferred into E. coli DH5α and freezer stocks were made.

In order to transfer the plasmids from E. coli DH5α into P. profundum a tri-parental mating is required. This involves mixing equal numbers of donor (DH5α), recipient (P. profundum TW30) and helper (carrying the F pilus genes) strains together, plating onto marine broth plates, incubating for 40 hours and
then re-suspending the cells and selecting for the correct antibiotic resistance: in this case resistance to rifampicin and streptomycin: \((P.\ profundum\ TW30\ has\ a\ chromosomal\ rifampicin\ resistance\ gene\ and\ pFL190\ carries\ a\ streptomycin\ gene).\)

Prior to 2009, mating experiments were carried out with moderate success in my hands: I was able to get several plasmids (including pFL190-GFP and pFL190-Bs) into strains of \(P.\ profundum\). However since this time no mating experiment has been successful. This has been a major setback in this project. At the time of writing I am trying to revive a fifteen year old freezer stock of \(P.\ profundum\ TW30\ complimented\ with\ pMN185\ (containing\ the\ toxRS\ genes)\ which\ has\ been\ kindly\ supplied\ by\ Doug\ Bartlett\).\ However, due to time constraints I am not able to report further on this here.

In an attempt to identify the cause of this problem I set about using different strains of \(E.\ coli\) as the donor strain, different strains of \(E.\ coli\) as the helper strain and using different types of growth media. I also supervised an Honours student (Sam Curran) who investigated these problems.

Figure 4.17: Gel image of PCR products confirming toxR/S inserts into pFL190. For each of the three colonies picked there was a positive insert of either toxR or toxS. The toxR PCR product has a predicted size of 848bp the toxS a product of 560bp.
Sam’s project was to produce a strain of *P. profundum* which was expressing a fluorescent protein. In order to do this we selected 15 different plasmids with a range of fluorescent protein genes, origins of replication and antibiotic markers. During the early stages of Sam’s project our stock of Marine Broth ran out and, due to supply issues, no more was available so we tested a series of different media and settled on ZoBell Media (10g special peptone, 5g yeast extract and 1l sea water) as this proved to produce the best growth and is the basis for Marine Broth.

However, even with the new media, mating experiments were not successful and Sam’s project concluded that an auxotrophic strain of *E. coli* as a donor strain which carried the F+ gene, and therefore did not require a helper strain, might possibly produce a successful mating. It was believed at the time that the double antibiotics were affecting the ability of *P. profundum* to grow on ZoBell Media and by instead relying on the auxotrophy of the *E. coli* the rifampicin could be removed from the selection media.

Six months later, when I started attempting to get pFL190-\textit{toxR} and pFl190-\textit{toxS} into strain TW30, I used *E. coli* strain S17-1 as both donor and helper strain, as it has a chromosomal copy of the F pilus genes. By this time a new source of Marine Broth had been obtained. The S17-1 combination did not yield any growth on double antibiotics. The only auxotrophic strain available to me at the time was WM3064, a DAP minus strain which requires diaminopimelic acid (DAP) supplements in order to grow. However this strain had an unexpected resistance to streptomycin (this should not be the case according to the strain’s genotype) and therefore it was not possible to get pFL190 into that strain. A single attempt at mating p519nGFP from WN3064 into *P. profundum* TW30 was unsuccessful; this plasmid has previously been successfully mated into *P. profundum* SS9.

Next I tried using different helper plasmids. pRK2013, pRK2073 and pVS104 all carry the F pilus genes required to facilitate conjugal transfer but no mating
experiment worked with any of these plasmids.

As a final attempt to get pFL190 into TW30 pressure was used as a selective agent. In this experiment single and double antibiotics were used in both liquid and solid media. The liquid media was pressurised at 28MPa and the solid media kept at 0.1MPa. Those liquid cultures with just streptomycin and TW30 (2 controls lack the recipient strain) all grew at 28MPa, however no cultures with streptomycin and rifampicin grew. At 0.1MPa no cultures grew on either single or double antibiotics.

All of these findings point to the conjugal transfer failing: the plasmid is not moving from the donor strain to the recipient strain. As my mating experiment problems coincided with the change of media during 2009, I can only assume that something in the formulation of the new Marine Broth is affecting the formation of the F pilus, or is missing which is required for the formation of the F pilus. After consulting with the makers of Marine Broth (DIFCO) they confirmed they had changed the formula but would say no more than it was a change in their special peptone supply.

4.7 The Role of Compatible Solutes in Adaption to Growth at Pressure

In addition to measuring the growth rates and yields, extensive attempts were also made to identify and measure intracellular concentrations of compatible solutes.

4.7.1 Solute Accumulation in *P. profundum*

In a previous study using NMR by Martin *et al.* [53] it was shown that as pressure is increased *P. profundum* SS9 accumulates high concentrations of solutes within its cells. These authors also showed that *P. profundum* SS9 increases its intracellular solute levels as the external salt concentration is increased. The
compatible solutes of choice for *P. profundum* are alanine, betaine, glutamate and β-hydroxybutyrate, with β-hydroxybutyrate levels being increased at both high pressure and salt concentrations.

TMAO (Trimethylamine N-oxide) is a solute accumulated by many marine vertebrates, including most elasmobranchs (sharks and rays). TMAO has been found to be able to aid in the folding of proteins at elevated pressures [171, 172, 173]. This study [173] was carried out in the lab of Paul Yancey where a liquid chromatography method was used to separate folded and unfolded proteins. This allowed Yancey *et al.* to show that TMAO actively helps fold denatured proteins.

Several different methods are available for determining intracellular solutes concentrations. Martin *et al.* used an NMR method while other labs have used HPLC techniques. In my work I have made use of both techniques.

**HPLC**

High performance liquid chromatography (HPLC) separates out complex solutions via the differential interactions of molecules in the sample with the column through which the sample is passed. The column is referred to as the stationary phase and the liquid running through the column is called the mobile phase. There are two categories of HPLC: forward phase and reverse phase. Forward phase HPLC can be further divided into HILIC, size exclusion and ion-exchange variants. Once separated the molecules coming off the column can be identified either by mass spectrometry or by comparison to known standards.

The main factors which affect chromatography are: the stationary phase chemistry (which is discussed in detail below), the mobile phase composition, the mobile phase flow rate, and the column pressure and temperature. The flow rate is dictated, in part, by the column materials particle size as well as by the dimensions of the column itself. Flow rate and column material particle size also affect the pressure within the column, as does the nature of the mobile phase: higher organic solvent concentrations result in lower column pressures. Fluctuations in pressure
are a sign of damage to the column or to the HPLC instrument. Precipitation of the sample onto the column will cause a steady increase in pressure as well as fluctuations in pressure. Damage to the piston seals or to parts of the check valve will also cause fluctuations in pressure. Temperature affects the binding of some compounds to some column materials so it is important to ensure these are controlled and constant throughout an experiment.

In reverse phase HPLC, the most commonly used type of HPLC, samples are dissolved in water (sometimes with the addition of salts, acids or small amounts of organic solvents to increase peak separation) and injected onto a highly hydrophobic column: normally an 18C column with silica beads coated with C18 carbon chains. The sample is eluted off the column with an increasing concentration of organic solvent in the mobile phase. The different hydrophobicity of the compounds within the sample which allows them to be separated: more hydrophobic compounds bind more strongly and take longer to elute off the column. At high water concentrations the hydrophobic regions of the sample molecules bind to the column and in order to elute the sample off the amount of organic solvent in the mobile phase is increased. This solvent competes for access to the hydrophobic column so weakly bound molecules come off the column first. More tightly bound molecules come off at increasing solvent concentrations.

Reverse phase HPLC is used to separate amino acids, allowing protein identification [174, 175], detection of drugs of abuse [176], and food safety testing [177] among other applications. However it is not suited for the separation of highly hydrophilic compounds such as compatible solutes; for these compounds forward phase HPLC variants are needed.

In forward phase HPLC the sample is dissolved in a high concentration of organic solvent and injected onto a column with a totally different chemistry to the 18C columns used in reverse phase HPLC. The types of column chemistry used in forward phase HPLC vary widely depending on the application. Here, I will discuss HILIC (Hydrophilic Interaction Liquid Chromography [178]) columns
which were used in the current study. HILIC columns tend to be made of silica beads. In most cases, but not all, these are coated with highly hydrophilic groups such as amino (NH$_2$), Polar-Diol (-OCH(OH)CH$_2$OH) and sulfobetaine ((CH$_3$)$_2$S$^+$-CH$_2$-CO$_2^-$) groups, which separate via positive charge, negative charge or zwitterionic interactions respectively. Because most compatible solutes are zwitterions any of these columns are suited to separate these compounds. Because these columns are made of silica they should be operated at acidic pH so that the silica does not dissolve in the mobile phase over time.

HILIC has an additional advantage over reverse phase HPLC in that it is compatible with Electro-Spray Ionisation (ESI) mass spectrometry due to the volatile nature of the organic solvents used and the low concentrations of water in the mobile phase [179]. This means that ESI MS-MS, a more sensitive form of MS, can be used to identify unknown compounds eluting from the column, making this a very powerful analytical tool.

NMR

An alternative method to HPLC for the identification of compatible solutes is to use Nuclear Magnetic Resonance (NMR) spectroscopy. This method uses electromagnetic pulses, of a specific frequency, to excite nuclei of a specific type in the sample which in turn causes a release of energy (in the form of electromagnetic pulses). This released energy has a specific resonance unique to that nuclei. The resonance is also affected by neighboring nuclei which allows bonds between atoms to be identified and the structure of molecules to be built up during the data analysis. NMR is used to identify the structures of molecules ranging from small molecules to large proteins [180, 181].

Many different types of nuclei can be examined with this method. For biological purposes the most common are $^3$H, $^{13}$C, $^{14}$N and $^{31}$P. By using this combination of 4 nuclei it is possible to identify all the major groups of molecules in biological systems.
Because the NMR pulse excites every nuclei within the sample there is a very low signal to noise ratio, so that many thousands of pulses are needed to get reliable data. The nuclei above are not the most common isotopes found in nature: by artificially adding these isotopes to a growing culture they can be incorporated into the cell in greater amounts, improving the signal to noise ratio and making identification easier. Unfortunately these isotopes are very expensive, reducing the cost effectiveness of this technique.

NMR Studies on *P. profundum*

During the initial phases of this project I used NMR to identify solutes accumulated in *P. profundum* SS9, in an attempt to replicate, as closely as possible, the experiments of Martin *et al.* To this end I grew *P. profundum* SS9 in Marine Broth at 250mM and 500mM NaCl and at 0.1MPa and 28MPa. I extracted the compatible solutes using 70% ethanol followed by sonication and re-suspension in 10% deuterium oxide (D$_2$O). These samples were then placed into a Bruker 800 MHz NMR spectrometer with a cryoprobe attached, where $^1$H and $^{13}$C spectra were obtained. At no point were peaks corresponding to those identified by Martin *et al.* found. This may have been due to the low concentrations of the solutes within the sample. The largest peaks observed turned out to be from the HEPES buffer used in the culture medium. This is illustrated in the $^1$H spectra shown in Figure 4.18. Additional peaks to those attributed to HEPES were observed but no positive identification was possible with this data.
Figure 4.18: Proton (¹H) NMR spectra for HEPES buffer and *P. profundum* SS9 extracts from 0.1MPa and 28MPa. In the *P. profundum* samples the peaks corresponding to the peaks in the HEPES buffer sample indicating that this is the major component of these samples. Other peaks can be seen elsewhere in the sample indicating other compounds are present but these were unidentified.

These initial experiments highlighted three critical issues. Firstly, that the concentrations of solutes in my sample were too low to give a good noise to signal
ratio. Secondly, that my samples had to be “washed” in order to remove as much of the HEPES as possible, and finally that the cost of running NMR on each sample was too high to make this analysis effective. Each sample took 14 hours to run at £4 per hour. For two pressures and two NaCl concentrations for four strains in replicates of 3, 144 samples needed to be run in total. This meant that the cost of the experiment would be close to £8000, making it prohibitively expensive. It was for this reason, more than any other, that it was decided to use HPLC as an analytical method for identifying and quantifying the solutes accumulated in \textit{P. profundum}.

**HPLC Analysis of Compatible Solutes in \textit{P. profundum}**

Once the decision to use HPLC had been made a column needed to be selected. Because all the compounds of interest are small, highly soluble molecules it was unlikely that any retention on a standard C18 column would occur. This meant that another type of column was needed, with a different column chemistry. As mentioned above, HILIC columns are able to separate hydrophilic compounds so these types of column were investigated.

Several groups have used a Grom Sil Amino PR1 column to quantify levels of ectoine, a common compatible solute produced by many bacteria in response to changes in NaCl concentration [182, 183]. As the partial genome of \textit{P. profundum} 3TCK indicates there is an \textit{ectABC} pathway for the production of ectoine in this strain this column appeared to be ideal for my needs. After getting advice from the UK suppliers (Grace Discovery Sciences) of this column I was assured that it was able to separate alanine, betaine, $\beta$-hydroxybutyrate and glutamate as well as ectoine.

To test this the column was set up on an open access LCMS in the School of Chemistry. Standards of the compatible solutes proline, betaine, ectoine and $\beta$-hydroxybutyrate were made up in 80% acetonitrile and injected onto the column. Proline and ectoine were included as these are common compatible solutes in
other *Vibrionaceae* bacteria. In order to detect the solutes a UV detector was used at a wavelength of 210nm MS data was also obtained.

Figure 4.19: Chromatograms of standards run at 80% acetonitrile on the Chemistry open access HPLC.

As shown in Figure 4.19 I obtained good separation between all compounds. However $\beta$-hydroxybutyrate was not observed to elute off the column. The reason for this is unknown as $\beta$-hydroxybutyrate elutes at approximately 2.5min in 80% acetonitrile 1ml/min flow when run alone on the column (See Figure 4.20).

As the initial HPLC pilot study was quite successful I decided to continue with this method. In order to do this I obtained the use of a Thermo Surveyor HPLC from Prof. Alastair Aitken for no costs other than the consumable materials and solvents I would be using. This allowed me to run many more samples than was possible on the open access instrument in chemistry.
I set about running standards at different concentrations, creating standard curves which would be needed to quantify the solutes at a later date. This data is shown in Figure 4.20. In this experiment proline, betaine, ectoine and β-hydroxybutyrate were all run at 80% acetonitrile and the area under the peak measured. It should be noted that the peak shape of ectoine is different on this instrument.

Measurement of the area under the peak allows for the quantification of the compound being eluted off the column. Standard curves for proline, betaine, ectoine and β-hydroxybutyrate are shown in Figure 4.21: this data would allow me to quantify the solutes in the bacterial extracts.

Figure 4.20: Chromatograms of standards run at 80% acetonitrile on the Thermo Surveyor HPLC. Each standard, Betaine, β-hydroxybutyrate, proline and ectoine was run under identical conditions at different concentrations of standard. The β-hydroxybutyrate peak is not the ideal shape this is likely due to poor mixing of the mobile phase by the HPLC instrument as the samples are injected.
Figure 4.21: Calibration curves for standards run at 80% acetonitrile on the Thermo Surveyor HPLC. Betaine, proline and $\beta$-hydroxybutyrate all have straight lines indicating a direct correlation between concentration of standard and peak size. Ectoine has a curved line indicating a non-linear correlation between concentration and peak size, this may be due to the leading edge of the peaks seen in Figure 4.20.

Unfortunately, shortly after starting to run my extracts from *P. profundum* the Thermo Surveyor HPLC developed a technical problem. This breakdown was likely due to the lack of maintenance of the instrument during its long period (7 years) in storage prior to my gaining access to it. A Thermo engineer managed to localize the problem to the autosampler but was unable to identify whether the problem was mechanical, electrical or software based.

As the Thermo Surveyor HPLC was now decommissioned, the project remained on hold for almost a year while an alternative HPLC was sought.

During the 2nd half of 2009 I gained access to a new Dionex Ultimate 3000...
HPLC which was purchased by the School of Physics. This system lacked an autosampler so each sample had to be manually injected onto the column.

After initial tests with this system it became apparent that eluting the samples off the column using a gradient from 95% to 50% acetonitrile over a 20min period would allow for better separation of my samples and thus make identification and quantification easier. The set up was adapted so that the sample was diluted in 95% acetonitrile, injected onto the column which had already been equilibrated with 95% acetonitrile, and allowed to bind for 2min. This 2min period was intended to flush any salt and non-binding compounds off the column (the column volume is 1.6ml) prior to eluting the sample. After 20min the acetonitrile concentration was raised back to 95% preparing the column for the next sample. This gave a total run time of 30min for each sample.

The strains to be analysed were SS9, TW30 (toxRS mutant) and 3TCK. Each strain was grown in MOPS minimal medium in 5ml samples, at 250mM and 500mM NaCl and at 0.1MPa and 28MPa in triplicate. The cells for each sample were collected, dried, the solutes extracted with 70% ethanol and sonication and the dried extract diluted into 95% acetonitrile. 25µl of this solution was injected onto the column. Each sample was run in triplicate and no attempt was made at this stage to keep salt levels or pH between samples constant.

Upon running the SS9 0.1MPa and 250mM NaCl samples it became apparent that the replica samples were not running consistently. This is shown in Figure 4.22. The peak shape, under identical conditions should be the same for each replicate of the same sample, as should the retention time. Ideally there should be no more than 5% difference between samples in terms of retention time. Differences in peak shape and/or retention time between identical samples are indications of variations in the running conditions.
Figure 4.22: Representative data for solutes accumulated in *P. profundum* SS9. The last peak (co-eluting with glutamate) has too large a variation in replicates C1-C3 and between samples A-C for a positive identification. The variation in peak shape and size is also worrying and indicates problems with conditions on the column.

Figure 4.22 shows three different samples (A-C), with three replicates (1-3). For samples C1-C3 the retention time for the last peak (10.5-12min) shifts from 10.57min to 11.15min, indicating changes in the running conditions between these replicates of the same sample. Further more the retention time of this same peak is different in samples A1-A3. These samples (A1-A3) have a peak retention time clustering around 10.87min and samples B1-B3, which cluster around 10.50min, but less tightly than samples A1-A3. The peak shapes for each sample are also different. Variability in the retention times and peak shapes are observed throughout all the 108 samples making qualitative analysis hard and quantitative analysis impossible. The problem with the varying retention time
suggested that the column was not able to consistently retain the samples which is why this problem is not observed in the standards. As HPLC identification (without the use of MS) relies on the comparison of sample retention times (and peak shape) to standards, repeatability is essential. After several conversations with the column manufacturers the problem of retention time was believed to be due to the presence of varying salt concentrations within the different samples. As varying concentrations of NaCl are an intrinsic part of this project there was no way to control this other than by adding a salt to the mobile phase.

In an attempt to overcome the problems of varying retention times and peak shape, buffers were added to the mobile phase and the pH was controlled. The types of buffer which are compatible with high concentrations of acetonitrile are very few; those commonly used are formate and acetate buffers. Ammonium formate at pH3.5 and pH5.0 was run as a mobile phase at a final concentration of 5mM in 80% acetonitrile. Under both conditions standards were run but showed no improvement in retention variability and, more worryingly, pressure fluctuations were well above the normal range of ±1-2Bar, as shown in Figure 4.23. Pressure fluctuations are caused either by a precipitate forming on the column, blocking the path of the mobile phase or by the collapse of the stationary phase.
Figure 4.23: Pressure fluctuations in the HPLC due to the presence of buffer salts. Normal 80% isocratic running conditions without a buffer has a pressure of ∼92Bar with very little fluctuation throughout the 40min run (blue line). When 5mM ammonium formate pH5.0 is added to the mobile phase fluctuations are highly increased indicating blockages or precipitation on the column. This is highly damaging to the column and if left unchecked would result in the loss of the column.

The column was back flushed (washed with high water concentrations in the opposite flow direction to normal) for 24 hours at a low flow rate. The first 75min of this backwashing process can be seen in Figure 4.24. During this time pressure fluctuations decrease but do not return to normal variations of ∼1-2Bar.
Figure 4.24: Pressure fluctuations during backwashing of the HPLC column. Backwashing removes any solid material from the head of the column by flowing the mobile phase through in the reverse direction. As time goes on pressure fluctuations are reduced but not to the expected range of ∼1-2Bar. This backwash took a total of 24 hours at 0.4ml/min.

Next ammonium acetate at pH6.0 was tried as an alternative buffer. Within one run the pressure fluctuations had returned and I decided to halt to save the column from further damage. After consulting with the manufacturers of the column it appears there may have been a “miscommunication” between myself and their technical service manager regarding to the type of compounds I was trying to separate and the salt concentrations of my samples. While the Grom Sil Amino PR1 column is ideal for the identification and quantification of ectoine it is less suited to complex mixed samples, in the presence of salt.

Alternative column types were therefore sought. However, all HILIC type columns are affected by changes in salt concentration and retention of compatible
solute is unlikely on a reverse phase column.

Due to the lateness of this development in my PhD I decided to focus on the completion of other areas of my research rather than investigating and testing new column and mobile phase combinations.

4.8 Discussion

4.8.1 Developing High-Throughput Growth at Pressure

The main driving factor for the development of a high-throughput method of growing bacteria at pressure was the inadequacies of growing bacteria in bulbs and bags while at high pressure. In addition to this the large number of samples, time points and replicates made the task of generating high quality data for growth rates of bacteria at high pressure very daunting.

To be effective the sealed 96-well plates needed to be pressure resistant over many pressurization-depressurization cycles. Each well, within the plate, needed to be kept separate from the surrounding wells. The optical surfaces needed to be transparent but also flexible enough to transmit pressure and the adhesive used should not affect growth of the bacteria in any way.

The process of developing this method was very long and time consuming; several iterations of the design were tried using *P. profundum* SS9 and monitoring growth over several days. The final iteration of the sealed 96-well plates, as shown in Figure 4.3 are superior to bags and bulbs in all key areas mentioned above.

The sealed 96-well plates are able to transmit pressures up to 60MPa, only being limited by the pressure vessel used in this study, they function at temperatures between 9°C and 42°C and they have been tested through at least 20 pressurization-depressurization cycles before showing signs of wear. Due to the fermentation of glucose during growth CO$_2$ gas would come out of solution in some samples after depressurization causing small bubbles to form within some
wells of the plate, if kept out of the pressure vessel for extended periods (greater than 60 min) this could cause the adhesive film to stretch. However, because of the fast read times of the plate reader and the fact that a maximum of 10 plates were cultured at once this effect was only seen after the plates were removed from the pressure vessel at the end of the experiment.

The only drawbacks of using these 96-well plates are that each well, whether used or unused, needs to be filled, making it less suited for the growth of single cultures, and the clouding of the seals over time. Fortunately this clouding can be measured and corrected with ease post data acquisition.

Because of the unique expertise of Hugh Vass it may be possible, in the future, to build a pressurised 96-well plate which can be incubated in a plate reader, therefore removing the need for a pressure vessel. This would be a very complex design: pressure would need to be spread across the plate in a uniform manner, the dimensions of the plate would have to match those of a standard 96-well plate in order to fit the plate reader, and each well would need to be kept separate. Using the piston design of the pressure cells it may be possible to build “lid” of 96 pistons which can be placed in a titanium 96-well plate. This can then be pressurised in a vice-like device and the lid locked in position by several tens of bolts across the plate. In order to measure internal pressure several wells could be filled with different types of liquid crystal so at a given temperature the pressure is always known.

4.8.2 Growth of *P. profundum* Strains

Having developed the high throughput growth method I set about measuring the growth rates and growth yields for *P. profundum* strains over an range of pressure and salt conditions. Because of the high throughput growth method I was able to include eight replicates for eleven different salt concentration at four pressures.

There is an interplay be the pressure and salt tolerance in the *P. profundum*
strains I have looked at in this thesis. For \textit{P. profundum} SS9 and 3TCK the salt tolerance is dependent on the pressure at which the train is growth. The pressure tolerance for these strains is also dependent on the salt concentration at which they are grown. This seems to suggest that when \textit{P. profundum} is subjected to one stress (either pressure or salt) it is less able to cope with other stresses. However, this picture seems to be more complicated when growth rates are considered.

For \textit{P. profundum} 3TCK the observed growth rate and growth yield are similar in pattern with the ability to gain biomass over a wide range of salt concentrations but only over a limited pressure range. The growth rate map and the growth yield map for \textit{P. profundum} SS9 were expected to be qualitatively similar, as with \textit{P. profundum} 3TCK. However this was not the case. It was expected that \textit{P. profundum} SS9 would be able to gain biomass over a moderate salt range, but have a requirement for pressure. This is seen in the growth yield data but not the growth rate data. The growth rate map shows three optima for the rate of the production of biomass. This suggests that the interplay between pressure tolerance and salt tolerance has a greater effect on the rate of biomass production than the efficiency of biomass production (i.e. the number of cells per unit glucose). Interestingly at 50MPa \textit{P. profundum} SS9 maximizes growth rate at low and high salt concentrations. This might suggest some kind of evolutionary tradeoff between growing fast and using resources efferently depending on the pressure and salt concentrations of the surrounding environment.

\textit{P. profundum} SS9 was isolated from an amphipod homogenate [4], perhaps it is a commensal, symbiotic or pathogenic organism which has evolved to respond to the host organism as well as the surrounding environment. This may go some way to explain the difference in salt and pressure tolerance of the two strains but this hypothesis would require more data.

It is interesting that, for \textit{P. profundum} SS9, the pressure optima, for either growth rate or growth yield, do not match that found by DeLong [4]. It was
expected that SS9 would have an optimal growth peak at 28MPa, instead I observed maximum growth yield at 10MPa and maximum growth rates at 10MPa and 50MPa. These effects are likely to be due to the medium in which the experiments were carried out. In DeLong’s experiments Marine Broth was used, where as in my experiments MOPS minimal media was used. MOPS minimal media only supplies the essential elements for growth, all non-essential components need to be synthesized by the bacterium itself. It is known that pressure sterilization is more effective in PBS and other buffers than it is in complex food stuffs and growth media [80]. What is observed here, I believe, is a similar effect: the rich Marine Broth is able to supply ready formed compounds for growth at pressure, without which the ability to grow at pressure is affected. The would mean that the cell has to spend more energy protecting itself from the effects of pressure rather than producing biomass.

For *P. profundum* SS9 the effect of added external compatible solutes, either betaine or TMAO, shifts the growth yield optima to both higher pressures and higher salt concentrations. This fits the hypothesis that the addition of compatible solutes to the growth medium improves pressure and salt tolerance. Furthermore it is interesting that a shift in pressure and salt optima is observed, not an extension. An extension of the growth optima would suggest the *P. profundum* SS9 is able to regulate the uptake of these compounds, as at lower pressures and salt concentrations it would be excluded from the cell and have no effect on growth. However this is not the case; it appears that these compatible solutes are not regulated in this way (or poorly regulated) and the accumulation of these compounds in the cell does affect growth at lower pressures and salt concentrations.

The addiction of external TMAO is not enough to adapt a non-pressure adapted organism to growth at high pressure. This is observed by shift of growth yield optima for *P. profundum* 3TCK to higher salt concentrations but not higher pressures. This observation is further complicated by the fact that the addition of
betaine has a negative effect on growth yield over all but an extension of growth at higher pressure is observed.

The observations of complex growth rate verses growth yield maps, the differences in response of different strains to the addition of external compatible solutes and the differences between rich and minimal growth media strongly suggests there is a link between pressure adaption and adaption to high salt. However this is a complex relationship and teasing out the exact mechanisms is not going to be simple and will require more investigation.

In addition to investigating the effects of salt and pressure on the growth of \textit{P. profundum} wild-type strains, I also investigated the effects of pressure and salt on the growth of a \textit{toxRS} mutant, \textit{P. profundum} TW30. In \textit{Vibrio cholerae} ToxR regulates a large number of genes on response to environmental stimuli, it has also been shown that in \textit{P. profundum} ToxR plays a role in pressure adaption.

The growth rate and growth yield maps for this mutant strain are very different to that of the parent strain, \textit{P. profundum} SS9. This shows that ToxRS plays an important role in the response to pressure and salt. In particular the pressure optimum is decreased and the salt range narrowed. The “X” shape observed for the growth yield is very interesting and deserves further investigation, however, due to time limits I am not able to do this myself.

ToxR is a regulatory protein, its function is modified by ToxS (and in \textit{V. cholerae} ToxT), so therefore it is important to isolate the effects of ToxR and ToxS. To do this I attempted to clone the individual genes back into TW30. This experiment was unsuccessful due to technical problems of getting the plasmid constructs into this strain. I believe that the problems I came across in this experiment have two sources: firstly \textit{P. profundum} is a difficult bacteria to work with and secondly the change in growth media had a negative effect on the mating process. Having had conversations with Doug Bartlett it appears his lab had similar problems when he started working with \textit{P. profundum}. They overcame these problems by using a higher concentration of agar in the medium (17g/L
instead of 15g/L) and ensuring the plates were very dry during the mating process. As to why this would have an effect on mating is not clear, but it appears that the lack of water causes the bacteria to become stressed and either more accepting of extracellular DNA or better able to form pilus with the *E. coli* strains. Doug Bartlett also suggests using a filter paper matrix rather than doing the mating directly on the agar surface.

Unfortunately, these conversations with Doug Bartlett only took place in September 2010, too late to act on this advice.

### 4.8.3 Measuring Compatible Solutes

One of the original aims of my PhD was to investigate the solutes synthesized *de novo* by the different *P. profundum* wild-type strains. The lab of Doug Bartlett performed a similar experiment using only *P. profundum* SS9 and not differentiating between *de novo* synthesis and uptake from the growth media. There are essentially two ways to carry out this experiment, the first is to use NMR as was done by the Bartlett lab. The second is to use HPLC and this is the method I chose to use having discounted NMR on the basis of cost.

As it turns out this was the wrong decision.

Reverse phase HPLC is very common, it is robust against changes in salt concentrations of the samples and due to its ubiquitous nature there is a large amount of technical support available. However the ability of reverse phase HPLC to separate the compatible solutes being looked for is somewhat limited. I chose to use HILIC HPLC as this offered greater separation of the compatible solutes I sort to identify. At the time of this decision I was not aware of the huge effects salt would have on the retention of these compatible solutes and the effect that would have on analysing the data.

In order to answer the original aim of this project I would need to totally redesign this experiment. Firstly I would aim to use NMR through out the
experiment. This technique is very robust, it is not affected by salt concentrations of the sample and the data analysis can be carried out using the correct software.

This then raises the question of cost. While cost is something not normally considered by a PhD student it is pertinent to this experiment due the money involved. One way of driving down costs is to re-design the experiment with fewer replicates and fewer conditions, this is not ideal as it increases statistical error in the data but may be acceptable should the differences between conditions be large enough. Another way of reducing cost would be to work as a collaboration with the chemistry department. In exchange for one or more names on any resulting paper it may be possible to get training to use the NMR instruments removing the cost of an operator. This would have also allowed me to increase my skill set.

Should cost be an insurmountable obstacle then HPLC is still an option. In this situation I would trial a dual column approach first, whereby the sample is run on a reverse phase column to separate the compatible solutes from the salt in the sample. The compatible solute fraction could then be collected, dried and run on a HILIC column in order to separate out the compatible solutes. Instead of an amino-type column, such as the one used in this study, I would trial a sulfobetaine type column. These columns have the advantage that they can be run at very low pH, meaning that the compatible solutes within the sample will always be charged and run in the same way.

It is my hope that, at some point in the future, another PhD student will be able to use the samples I have made and the answers to the original questions as to whether *P. profundum* produces compatible solutes *de novo* or not is answered.
Chapter 5

High Pressure Growth Screening

5.1 Introduction

In order to ascertain which genes are important for growth at pressure in *E. coli* the newly developed high-throughput growth method was used to screen a library of *E. coli* mutants for the ability to gain biomass at the normally permissive pressure of 30MPa. Mutants which proved unable to grow at this pressure were considered to have a gene deletion important in pressure adaptation and their genotypes and phenotypes were investigated further. To my knowledge this is the first systematic screen of *E. coli* deletion mutants for growth in this pressure range. However, there have been several screens of transposon mutant libraries at lethal pressures (>200MPa). These have identified genes which are essential for outgrowth after pressure treatment. Promoter fusion libraries have also been screened for the induction of a reporter gene (GFP in this case) in cells treated with very high pressures for short periods [71, 70, 69, 184].

The ability of *E. coli* to grow at elevated but permissive pressure has not been investigated to any large extent; the majority of previous research has focused on sterilisation of *E. coli* using much high pressure (>200MPa). This interest in high pressure sterilization is due to the improved texture and quality of foods processed
in this way as compared to heat or chemically treated foods. It is known that *E. coli* is able to complete, but not initiate, DNA, RNA and protein synthesis at pressures above 60MPa [72]. At lower pressures the initiation of macromolecular synthesis is able to occur but at a reduced rate compared to that at 0.1MPa. Microarray analysis of *E. coli* grown at 0.1MPa, 30MPa and 50MPa was carried out by Ishii *et al.* [185]; this showed that in exponential phase 469 and 698 genes had significantly altered expression at 30MPa and 50MPa respectively. These genes were spread across the genome and included all gene function categories. However, microarrays are inherently noisy and can be unreliable. One method of reducing noise is to tag the cDNA with both green and red labels in separate experiments. Ishii *et al.* state that this was not done in their study, making the results less reliable. In another study by Malone *et al.* microarray analysis of *E. coli* treated at 100MPa for 15min revealed that 36 genes had altered gene expression when compared to 0.1MPa [186]. These genes fell into five groups: stress response, thiol-disulfide redox system, Fe-S cluster status, spontaneous mutation and miscellaneous.

It is also known that RecD plays a role in pressure adaptation and plasmid replication at pressure in *E. coli* [187] however, its role is not understood fully. In a study by Doug Bartlett’s group a library of *P. profundum* SS9 deletion mutants was screened for growth at 30MPa, out of this screen a *recD* mutant was identified as being pressure sensitive. When the *P. profundum* SS9 *recD* was transformed into an *E. coli recD* mutant this strain gained pressure resistance at 31MPa.

### 5.2 The Keio Collection

*E. coli* is quite possibility the most studied organism on the planet, rivaled only by *Homo sapiens*. This has led to the development of a very large platform of tools which allow different aspects of this organism to be explored. One such tool is the Keio Collection - a collection of *E. coli* k12 strains containing every
The Keio collection was produced by Barry Wanner and collaborators in a systematic manner thanks to the genome sequence of *E. coli* K12, which was used to identify all possible open reading frames (ORFs) within this organism. Of the 4453 ORFs in the *E. coli* chromosome 4296 were targeted for deletion, resulting in 3985 successful deletions. These mutants were produced independently at least twice and verified via sequencing of the deletion site. Those genes whose deletions were unsuccessful were considered essential for the replication of *E. coli* under 'normal' conditions. By normal conditions the creators of the Keio collection mean aerobic growth on LB medium at 37°C. Each Keio mutant has a carefully designed, in-frame gene deletion comprising a kanamycin resistance gene flanked by FRT sites which allow for easy excision of the kanamycin resistance gene. The Keio Collection is distributed, freely, by the National Institute of Genetics (NIG), Japan, and comes as a set of 45 96-well plates, which are stored at -80°C.

The Keio collection mutants were designed to create in-frame (nonpolar) deletions upon elimination of the resistance cassette, to keep downstream effects of the gene deletion to a minimum.

The Keio collection now forms part of an initiative (www.ecolicommunity.org) to collect and make freely available all information on *E. coli*. Through this portal it is possible to search for information on any *E. coli* gene in 24 separate databases giving details on gene expression levels, protein localization, KEGG pathway analysis, COG analysis, GFP tagging data, proteomics, metabolomics and genome sequence data. This is a very powerful tool bringing together much of the relevant information on *E. coli*.

To create the Keio Collection mutants a recently developed double crossover method is used. This relies on the bacterium taking up extracellular DNA into its cell, the binding of the extracellular DNA to the chromosomal DNA and via the DNA recombination pathway the extracellular DNA is intergraded into the chromosome. Previously the production of gene knock outs relied on plasmid
based “suicide vectors”. These are plasmids containing sequences homologous to the ends of the region of the genome to be deleted and an origin of replication foreign to the strain in which the deletion is to occur. These plasmids are taken up by *E. coli* but unable to replicate, therefore, by selecting for the antibiotic encoded on the plasmid, the plasmid becomes integrated into the chromosome at the site of homology. The limitation to using non-plasmid based systems for gene deletions is due to the high efficiency of *E. coli* at degrading linear DNA, meaning that linear DNA would be degraded before being integrated into the chromosome. Datsenko and Wanner [188] developed a system allowing PCR products to be used directly in the knockout of regions of DNA in *E. coli*, allowing the time consuming step of producing plasmids to be removed from the knockout procedure.
Figure 5.1: Strategy for the knock out of *E. coli* Genes used in the production of the Keio Collection. Targeting of Kanamycin selection marker, Step 1 generation of PCR product with homology to the region being knocked out, Step 2 transformation of PCR products into strain carrying λ Red system, Step 3 selection for Kanamycin resistant transformants. FRT site scars after the removal of Kanamycin resistance marker. Taken from [56]

Key to this method is the production of designed PCR products, comprising an antibiotic selection marker, flanked by flip recombination target (FRT) sites and wings of homology to the area of the chromosome immediately outside of the ORF to be deleted. This PCR product can be transformed directly into BW25113 cells, a strain of *E. coli* W3110, and selection for the antibiotic results in the integration of the PCR product into the chromosome. The homology wings have to be between 50 and 70bp for efficient recombination.

The use of *E. coli* strain BW25113 is essential for this process to work efficiently. This mutant has 13 mutations which prevent it from degrading linear DNA and increases efficiency of chromosomal integration. Instead of using
the native *E. coli* recombination system, the Red recombination system is used (comprising $\gamma$, $\beta$ and *exo* from bacteriophage $\lambda$ carried on pKD46) which, upon a temperature shift from 30°C to 42°C, integrates the linear PCR product into the chromosome at the site of homology.

### 5.3 Initial Screening of the Keio Collection

I began with a non-quantitative, "proof of concept", screen of the Keio Collection, assessing by eye whether each mutant was able to grow at pressure. To carry out this screen each Keio collection plate was taken from the -80°C freezer, allowed to thaw slightly, replicated into 200$\mu$l LB supplemented with 30$\mu$g/ml Kanamycin and 25mM glucose and grown for 24 hours at 37°C and 200rpm. These starter culture plates were then replicated into fresh 96-well plates containing 382$\mu$l LB/Kan/Glucose. Once sealed using the method described in section 4.1, these plates were incubated at 30MPa, 37°C for a further 24 hours. After incubation each plate was inspected, by eye, for growth or no growth.

This non-quantitative screen highlighted 17 deletion mutants which showed no growth at 30MPa: *gpmI, yfgA, dedD, sufI, holC, tatB, ygbF, envC, priA, dnaT, atpF, dnaK, iscS, tolB, rimM, yhcB* and *rfaC.*

These mutants were re-screened, this time using a 96-well plate reader to quantitatively measure the final $\text{OD}_{600\text{nm}}$ along with controls: BW25113, *betA, fliC, hns, metC, recD* and *rpoS,* which were expected to have no defect in growth at 30MPa.
Figure 5.2: Growth yield pressure sensitive mutants identified in non-quantitative screen. The mutants identified in the non-quantitative screen were re-screened and the final $OD_{600\text{nm}}$ measured at 24h. The error bars show standard deviation for 4 replicates. The majority of suspected pressure sensitive mutants, in blue, have repeated well showing a consistent pressure sensitivity. Control strains are in red and parent strain in green.

The results are shown in Figure (5.2). Of these mutants it was decided that $atpF$, $dedD$, $dnaT$, $envC$, $gpmI$, $holC$, $iscS$, $priA$, $rimM$, $tatB$ and $yfgA$ were all bona fide pressure sensitive mutants at 30MPa and the others were not. However significant variability was present between replicate experiments as shown by the large error bars.

5.4 Quantitative Screen of the Keio Collection

After the positive results of the initial screening of the Keio Collection it was decided to carry out a second, fully quantitative screen of the whole Keio
Collection, measuring the optical density of each culture after pressurisation. This was possible due to the clear seals used for the pressure resistant 96-well plates and the availability of a plate reader to measure optical density (OD(600nm)) accurately. As before the freezer stock plates were replicated into 200µl LB/Kan/Glucose and these starter plates were incubated at 200rpm at 37°C overnight before being replicated into 382µl fresh LB/Kan/Glucose and incubated at the appropriate pressure for 24 hours. After this time the OD(600nm) of each well was measured in the plate reader without removing the seals. In addition to the 30MPa screen, a 0.1MPa and 50MPa screen were also performed. These additional pressure screens were added to allow comparison of non-stressed (0.1MPa), some-what stressed (30MPa) and highly stressed (50MPa) cultures. The rationale behind the 0.1MPa screen was to ensure that any mutants unable to grow at 0.1MPa were not counted as pressure sensitive. The 50MPa screen was added to allow the identification of possible pressure resistant mutants, able to grow better than the parent strain at higher pressures.

A series of control plates were also set up; these consisted of the parent strain (BW25113) and media only wells of 96 replicates each. These controls gave the maximum OD(600nm) for the parent strain at the three different pressures (for comparison to the mutant strains) and the average OD(600nm) for media alone. The average value for the media alone control (measured at 0.1, 30 and 50MPa) was used to blank the raw data for the rest of the screening data. This blank value increases over time, in a reproducible manner, due to the clouding of the seal adhesive (see Chapter 4 for details).

The results of this screen are shown in Figure 5.3. In this figure, the OD(600nm) reading for each well was binned into groupings of 0.1 and these were plotted as a histogram. The 0.1MPa cultures had an average final OD(600nm) of 1.400(±0.209), which is as expected. In comparison, the 30MPa cultures had an average final OD(600nm) of 0.949(±0.147) and the 50MPa cultures had a final OD(600nm) of 0.207(±0.140). This showed that an increase in pressure has an effect on the
population of mutants as a whole; i.e. as pressure increases the ability of *E. coli* to grow decreases. Interestingly, the 30MPa screen revealed some mutants with much greater than average pressure sensitivity. These mutants form a small peak close to an OD\(_{(600\text{nm})}\) value of zero in Figure 5.3. Before the screening I had thought that the 50MPa screen might produce some pressure resistant mutants; however this was not the case, these would show as a peak to the right of the main body of mutants as seen in Figure 5.3. This suggests that the effects of gene deletion are always deleterious in terms of ability to grow at pressure; no gene deletions were found which improved growth at prohibitive pressures. Gene deletion mutants which failed to grow at 0.1MPa were discounted when determining mutants for further screening. These mutants are *yniC*, *thyA*, *oxyR*, *setB*, *yfcX*, *yehD* and *stfE*. 
Figure 5.3: Histogram of data from quantitative screen. Cultures grown at 0.1MPa have an average of 1.400(±0.209) well within the expected range. Cultures grown at 30MPa have an average of 0.949(±0.147) which is significantly below the average for 0.1MPa cultures. Cultures grown at 50MPa have an average of 0.207(±0.140) significantly lower than 0.1MPa and 30MPa data. Mutants unable to grow at 30MPa are seen clustering around OD 0 (bottom left of chart).

One interesting question is whether the same mutants show enhanced or reduced growth, relative to the parent strain, at different pressures. If this is the case, then there should be a clear correlations in the final $OD_{(600nm)}$ values recorded at 0.1MPa and 30MPa and between 30MPa and 50MPa. These correlation plots are shown in Figure 5.4 and colour coded by COG classification of the deleted genes. No obvious correlation is present. This suggests that the physiology of the cell is quite different at different pressure so the relative effects of a given deletion are different at different pressures.
Figure 5.4: Correlation plot for quantitative screen of the Keio collection at pressure. There is no significant correlation for pressure sensitivity between 0.1MPa and 30MPa and between 30MPa and 50MPa.
Figure 5.5: Repeat OD data for selected pressure sensitive mutants, control strains and parent strain. Pressure sensitive mutants, blue, fall into two groups: absolute inability to grow at 30MPa and reduced growth at 30MPa, control and parent strains are all able to grow to a higher OD than pressure sensitive mutants. Error bars are standard deviation for four replicate cultures.

The 85 mutants which showed greatest pressure sensitivity at 30MPa were selected for further screening and combined in a single 96-well plate. In addition 3 control strains (recD, fliF and rpoS) and 4 replicates of the parent strain (BW25113) were also included in the plate. Using replicates of 4 plates these strains were tested for growth at 0.1, 10, 20, 30, 35, 37.5, 40, 45, 50 and 60MPa. The aim of this experiment was to characterise, in detail, the critical pressure at which these mutants become unable to grow. The results of these experiments, shown in Figure 5.6, show that different genes become essential for growth at different pressure thresholds. The parent strain, coloured black, grows well up to 45-50MPa, above which it abruptly fails to grow. In contrast the mutant

The functions of the deleted genes in these mutants fall into four groups. The mutants with the lowest pressure threshold are those genes involved in DNA replication and repair (dnaT, priA, holC and nusB). The next group of mutants lack genes encoding structural proteins including yfgA/rodZ and dedD. The third group of mutants lack specific genes encoding membrane bound proteins (tatC, tolQ, tolB and atpG) and the final group lack genes encoding proteins with unknown or other functions (iscS, rffT, ydaS and ybhH). This finding shows that DNA replication and repair in E. coli is very sensitive to changes in pressure, specifically the mutants lacking dnaT, priA and holC are all deficient in the machinery for replication of the lagging DNA strand (See below for details).
Figure 5.6: Average OD data for selected mutants at pressures from 0.1MPa to 60MPa. For each mutant or parent strain there is a pressure at which growth can no longer occur, for *bona fide* pressure sensitive mutants this is well below that for the parent strain, these pressure sensitive mutants are seen between 25MPa and 50MPa. Parent and control strains are in black and yellow respectively, these have high OD readings at lower pressures which start to decrease at \( \sim 50\text{MPa} \). Pressure sensitive mutants start to have reduced OD values from \( \sim 25\text{MPa} \). DNA replication/repare mutants (red) are most pressure sensitive along with cell shape mutants (green), mutants in membrane proteins (blue) and other mutants (orange) follow a similar pattern but at different pressures.

The results in Figure 5.6 suggest that different physiological functions become sensitive to pressure at different pressure thresholds. If this is the case, then the pressure threshold of different mutants should cluster according to their physiological function. To test this, I defined a pressure value, PCross, at which each of the curves in Figure 5.6 crossed the arbitrary value of OD0.2. This was regarded as the OD threshold below which there was no growth. Figure 5.7
shows distinct groupings of pressure sensitivity. These groups loosely correlate with gene function: those most pressure sensitive are DNA replication and repair genes, followed by cell structure and transport across the membrane.

Figure 5.7: PCross analysis of pressure sensitivity. Using the value PCross (the pressure at which the OD reaches a value of 0.2) it is possible to rank pressure sensitivity. Within these data there are four groups as highlighted.

In this study I have, in effect, measured the growth yield for each of the mutants at different pressures. However, if a mutant is particularly slow growing it might show up as an apparently low growth yield at 24 hours. In order to identify the those mutants which low growth rates the 88 mutants and 4 replicates of the parent strain were grown aerobically in the plate reader at 0.1MPa for 24 hours with shaking at 200rpm.

As shown in Figure 5.8 when there is no correlation between pressure sensitivity and growth rate at 0.1MPa. This suggests that the mutants identified
do indeed have a reduced growth yield at pressure, rather than just being generally slow growing.

Figure 5.8: Growth rates and PCross correlation for pressure sensitive mutant strains at 0.1MPa. Left panel: Each strain was grown in the plate reader at 37°C 200rpm, their OD$_{600nm}$ measured every 10min the gradient of the exponential phase measured and growth rate (k) plotted. Error bars represent standard error. Right panel: Correlation between PCross and growth rate for each mutant. As pressure sensitivity increases (lower PCross) growth rate is not affected as seen by the linear trend in the data points.

5.5 Complementation of Pressure Sensitive Mutants

In order to confirm the link between genotype and the pressure sensitive phenotypes observed, plasmids carrying the knocked-out gene were introduced back into selected mutant strains. These plasmids were obtained from the ASKA collection: a plasmid library designed to complement the Keio collection. This collection is made freely available by NIG Japan. Each plasmid consists of a pCA24n “backbone”, containing a chloramphenicol resistance gene (cat), a high copy number origin of replication and a $P_{T5-lac}$ promoter [189]. The open reading frame of interest is cloned into this backbone so as to be expressed from the $P_{T5-lac}$ promoter on addition of IPTG. The ASKA collection contains plasmids with all
the 4327 ORFs in *E. coli* W3110. Each ORF is available with, or without, an N-terminal GFP tag. For these experiments I used plasmids without the GFP tag. The mutants chosen for complementation were: *priA*, *dnaT*, *holC*, *yfgA/rodZ*, *dedD* and *tatC*.

Competent cells for each of the selected deletion mutants were made and transformed with purified plasmid DNA from the ASKA Collection, corresponding to the disrupted gene. With the exception of the *dnaT* mutant, all the plasmids were successfully taken up as shown by gain of resistance to chloramphenicol. As a control the parent strain, BW25113, was transformed with pCA24n carrying a GFP gene only.

As shown in Figure 5.9, the restoration of the disrupted genes in the 9 pressure sensitive mutants resulted in a pressure phenotype much closer to that of the parent strain than that of the deletion mutant. This data confirms the link between genotype and pressure sensitive phenotype.
Figure 5.9: Complementation of pressure sensitive phenotype at 30MPa for selected mutants. Each mutant was transformed with a plasmid from the ASKA collection corresponding to the deleted gene in the mutant strain. These strains were grown in sealed 96-well plates for 24h at 37°C and 30MPa. For each of the selected mutants pressure sensitivity was reduced after complementation with the deleted gene confirming that the observed phenotype is due to the deletion of said gene.

5.6 Genes Required for Growth at Pressure

5.6.1 DNA Replication and Repair

The first group of pressure sensitive mutants identified in my screens lack genes involved in DNA replication and repair; these are priA, dnaT, holC and nusB. PriA and DnaT are involved in the formation of the primosome, a complex of 7 proteins (DnaGBCT and PriABC) on the lagging strand of the DNA replication fork. The function of the primosome is to produce RNA primers for the replication of DNA in the 3’ to 5’ direction. PriA, PriB and PriC bind to the single
stranded DNA at the replication fork and sequester DnaB, DnaC and DnaT to form a pre-primosome. The binding of DnaG completes the primosome. This complex produces RNA primers of 1-10 bases on the lagging strand, allowing DNA polymerase III to attach and begin DNA replication.

A primosome lacking PriA is less well adapted to binding to single stranded DNA in order to recruit the DnaBCT complex, as reflected in a reduced growth rate for the priA mutant at 0.1MPa (Figure 5.8). The dnaT mutant also shows a reduced growth rate phenotype at 0.1MPa, which can probably be attributed to the reduced affinity of the DnaCB complex for the PriABC complex.

The third gene in this group is holC, a DNA polymerase III chi subunit. The chi subunit of DNA polymerase III attaches to the lagging strand of replicating DNA (while the tau subunit attaches to the leading strand), orientating the polymerase so as to allow the beta2 subunit to access the single stranded DNA for replication. The holC mutant also shows a slightly lower growth rate than the parent strain at 0.1MPa, as seen in Figure 5.8.

Taken together, these observations suggest that the replication of the lagging strand of the DNA is a physiological process which is highly sensitive to pressure. This process appears to become growth limiting at around 25MPa. The pressure sensitivity of lagging strand replication appears to apply to both the formation of RNA primers and DNA polymerisation from those primers.

### 5.6.2 Structural Proteins

The second group of genes which are essential for growth at 30MPa are involved in cell division, cell shape and cytoskeletal structure. These genes are dedD and yfgA/rodZ.

DedD is a protein which is involved in the formation of septa during cytokinesis [190], however the function of DedD is not fully understood as yet. Mutants lacking DedD often show a chaining morphology whereby daughter cells are
unable to efficiently separate after the formation of a septum. DedD, like FtsN, has a C-terminal SPOR domain, directing the protein to the periplasm and the forming septal ring. In a ΔftsN background dedD becomes essential under normal growth conditions [190]. The first step in the formation of the septal ring is the polymerisation of FtsZ. This recruits FtsA and ZipA onto which the remaining proteins attach. Interestingly it has been shown in vitro that FtsZ polymerisation is highly sensitive to pressure: at pressures of 40MPa FtsZ is unable to polymerise in vitro [191]. DedD interacts directly with the FtsZ ring [190] and may be required to enhance polymerisation at pressure and thus allow cell division to occur.

The cytoskeleton of a rod shaped bacterial cell is formed of a murein (peptidoglycan) sacculus, which maintains cell shape and internal turgor pressure. MreB is the only known bacterial actin like protein in *E. coli* [192]: it forms just below the cytoplasmic membrane in a spiral-like configuration. MreB is anchored into the peptidoglycan and functions in maintaining cell shape and facilitating chromosome segregation and cell replication [192]. There is no *mreB* mutant in the Keio Collection as this gene is essential for growth.

The gene product of *yfgA*, now reclassified as *rodZ* [193], is a bitopic membrane protein (having only 1 membrane spanning domain) predicted to have a CRO/CI-type DNA binding region [194]. RodZ has been shown to co-localize with MreB [193]. Cells lacking RodZ form large spheroids under normal growth conditions at 30°C but are not able to propagate at 42°C on any media tried [193]. Over expression of FtsZ in *rodZ* mutant strains is able to partly restore the wild-type phenotype.

These observations suggest that protein polymerisation, in particular that involved in septal ring formation, is a pressure sensitive physiological process. Mutants deficient in the proteins which facilitate this polymerisation are unable to grow at pressures above 35-40MPa.
5.6.3 Membrane Bound Proteins

The third group of pressure sensitive mutants lack membrane bound proteins. These are: tatC, tolQ, tolB and atpF. Other atp mutants are also affected by pressure but not to the same extent; the same is true for other tol and tat mutants but also to a lesser extent.

The twin-arginine translocation (tat) pathway comprises three proteins encoded by tatA, tatB and tatC with auxiliary genes tatD and tatE. The gene product of tatA forms a protein-conducting pore, the size of which is dependent on the protein which is to be exported ([195]). The gene products of tatB and tatC form a complex which plays a role in targeting the substrate for transport across the membrane [196]; TatC recognises a conserved targeting sequence of S/T-R-R-x-F-L-K [197].

As TatA, TatB and TatC are all essential for the correct functioning of the TatABC transporter it is interesting that the tatA mutant is not pressure sensitive. In the quantitative screen at 30MPa the tatA mutant had a final OD(600nm) of 0.740 which is comparable with the value for the parent strain of 0.638 ±0.058. This may be explained by the finding that TatE can perform some of the functions of TatA. Since the tatB and tatC deletions had no effect on cell lethality at 0.1MPa my results suggest that one of the proteins exported by this complex may be essential for growth at 30MPa. Two proteins which are exported by the Tat system are EnvC and SufI [198]. Mutants lacking envC and sufI were identified in the initial screen of the Keio Collection as being pressure sensitive, however these mutants showed little pressure sensitivity in repeat screens.

The Tol system comprises five proteins, TolQ, TolR, TolA, TolB and Pal. Mutations in any of these genes result in hypersensitivity to deleterious agents, release of periplasmic contents, formation of outer membrane vesicles at the cell surface and induction of capsule synthesis which results in a mucoid phenotype [199]. TolQ, TolR and TolA are cytoplasmic membrane proteins, TolB is anchored
into periplasmic side of the outer membrane and Pal is anchored to the outside of
the outer membrane. The TolB-Pal complex interacts with the TolQRA complex
in an energy-dependent manner which results in the uptake of type A colicins.
These colicins are toxic proteins which are active against *E. coli* and closely
related species[199]. It is not clear why the loss of TolB and TolQ leads to an
increase in pressure sensitivity. One explanation is that the Tol system takes
up compounds from the environment into the cell which are vital for growth at
pressure.

*atp*F is the fourth gene in the *atp*IBEFHAGDC operon. The primary function
of these gene products is to form the F1-ATPase membrane pump which generates
energy, in the form of ATP, by pumping protons across the membrane. No one
gene is essential for growth: deletions of all these genes are present in the Keio
collection. AtpF anchors the epsilon subunit (responsible for reduction of PPi
to Pi) to the A subunit bound in the membrane. Again, it is not clear how this
process is affected by pressure. However, it is know that in general that protein
complex formation is pressure-sensitive: it is possible that this mutant is already
impaired in function of the F1-Atpase complex, which is further exasperated by
the effect of pressure.

### 5.6.4 Common Features of Pressure Sensitive Mutants

The most striking common feature of all the pressure sensitive genes is that their
gene products all form part of larger complexes. PriA and DnaT form part of the
primosome, the Tol, Tat and ATPase complexes all form part of a large membrane
pores, DedD forms part of the septal ring and RodZ is associated with the MreB
cytoskeleton and the septal ring.

Another common feature of some of these gene products is their association
to cellular membranes. The Tat, Tol and ATPase systems form a pore within the
inner membrane, the septal ring, of which DedD forms a part is directly attached
to the inner membrane and the MreB cytoskeleton, onto which RodZ is attached, is directly under, and interacting with, the inner membrane.

These two observations suggest two possible modes of inactivation for these mutants. Firstly it is known that pressure affects the binding of large protein complexes [18]; this model can be used to explain the above affects. Secondly the lipid membrane has been shown to change configuration from a liquid crystal to a gel with pressure. The close association of some of these proteins to the inner membrane could be the cause of the above affects: if these proteins are unable to interact with the inner membrane they are unable to perform their function.

5.7 Microscopy of Pressure Sensitive Mutants

Several of the genes identified as being essential for growth at pressure are linked to cell shape or DNA replication and repair proteins. Such mutants often show cell shape phenotypes which are different from the normal rod shaped cells of the parent strain. For this reason I decided to image cultures of the pressure sensitive mutants after 24h at either 0.1MPa or 30MPa. To ensure sufficient bacterial cells for imaging, starter cultures were only diluted 1:50, giving a higher cell density. These cultures were then placed into 96-well plates, sealed and put into the 3 liter pressure vessel where they were incubated for 24 hours at 30MPa. These pressure treated cultures were then imaged using a Nikon Ti-U microscope with a 100x Plan Fluo oil immersion objective. Figure 5.10 to 5.15 show each of the pressure sensitive mutants identified in the original screen and its complimented counterpart after 24h at 0.1MPa or 30MPa.

The parent strain (BW25113), shown in Figure 5.10, shows normal rod shaped cells at 0.1MPa. This morphology is unaffected by the presence of pCA24n-GFP at this pressure. At 30MPa cells of the parent strain are slightly more elongated than at 0.1MPa, again this is unaffected by the presence of pCA24n-GFP.

For the DNA replication and repair mutants (priA, dnaT and holC), in
Figures 5.11, 5.12 and 5.13, the cells show an elongated (filamented) morphology at 0.1MPa. This is often indicative of the SOS response, whereby the cell cycle is halted (via the LexA/SulA pathway) due to DNA damage. This, in wild-type cells, allows time for the cell to repair any damage to the DNA before restarting the cell cycle. After 24h at 30MPa these mutants are hyper-filamented; cells are too large to fit into a single field of view. This is likely to be the reason why these cells are unable to grow at 30MPa. When the SOS response is activated the cell carries on producing new biomass, the cause of elongation, while the DNA is being repaired. Should the DNA damage not be repaired the filamentation will continue until the cell is no longer able to sustain its shape or the DNA damage is so severe that the cell is no longer viable. These effects are not seen in the complimented strains for these mutants, which show normal morphology at 30MPa, indicating that the effect is due to the deletion of the priA, dnaT or holC genes.
Figure 5.10: Images of *E. coli* strain BW25113 at 0.1MPa and 30MPa. At 0.1MPa the both the parent strain and the parent strain carrying complementation plasmid (pCA24n-GFP containing only GFP) have normal morphology. At 30MPa there is a small increase in cell length in both strains. Bar = 10µm
Figure 5.11: Images of *priA* mutant and complimented strains at 0.1MPa and 30MPa. At 0.1MPa *priA* mutant cells are filamented, an indication of the SOS response in *E. coli*, this morphology is corrected upon complementation. At 30MPa the *priA* mutant has increased filamentation and occasional irregular cell thickness. This morphology is reverted to that seen in the parent strain (BW25113). Bar = 10µm
Figure 5.12: Images of holC mutant and complimented strains at 0.1MPa and 30MPa. At 0.1MPa the holC mutant has a filamentosed morphology, suggestive of the SOS response in E. coli. This morphology is corrected with complementation via the pCA24n-holC plasmid. At 30MPa holC mutant cells become hyper-filamented indicating extreme induction of the SOS response, the complimented strain does not show this cell morphology. Bar = 10µm
Figure 5.13: Images of dnaT mutant strain at 0.1MPa and 30MPa. At 0.1MPa cells of the dnaT mutant are slightly elongated, suggesting expression of the SOS response, at 30MPa these cells become hyper-filamented, suggesting the SOS response is further induced. Due to the inability to transform these cells a complimented strain is not available. Bar = 10µm

It is unlikely that pressure is directly damaging DNA. However it has been shown previously that the restriction endonuclease Mrr cleaves DNA on pressure treatment at 100MPa triggering the SOS response [71]. lon mutants, which are hypersensitive to DNA damage caused by UV irradiation, also express higher levels of Mrr [163, 70] and have been show to be hyper-filamented at 50MPa [168]. Mrr is a type IV restriction endonuclease which creates double strand breaks in DNA as part of the normal DNA repair mechanism. Under normal conditions levels of Mrr are very low [200] but after treatment at 100MPa levels of Mrr were increased resulting in more DNA damage [71] which triggers the SOS response. It would be interesting to determine whether Mrr plays a role in the filamentation observed in these strains at 30MPa.

The cell shape mutants dedD and yfgA/rodZ have a array of cell shape
morphologies. For the \textit{dedD} mutant, cells at 0.1MPa are similarly shaped to the parent strain. Complementation at 0.1MPa does not affect this morphology. At 30MPa the \textit{dedD} mutant is highly filamented, a morphology similar to that of the \textit{priA} or \textit{holC} mutants. In the complimented strain this filamentation is somewhat reduced: the cells are forming chains which appear to have septa formed.

Figure 5.14: Images of \textit{dedD} mutant strain at 0.1MPa and 30MPa. At 0.1MPa the \textit{dedD} mutants has a regular rod-shaped morphology. As pressure is increased to 30MPa hyper-filamentation is observed. It is unlikely to be due to the SOS response as DedD is not involved in DNA replication or repair. This hyper-filamentation is likely to be due to the inability to form septal rings separating daughter cells after replication. complementation of this strain partly restores the parent phenotype, however chaining is still observed at 30MPa. Bar = 10\mu m

The \textit{yfgA/rodZ} mutant forms large spheroidal cells at 0.1MPa as expected from the literature [193]. This morphology is somewhat restored to rods in the complimented strain at 0.1MPa. However, somewhat paradoxically, the morphology of this mutant is very similar to that of the parent strain at 30MPa.
regardless of complementation. One hypothesis might be that another protein, possibly MreC (which is 2.91 fold induced at 30MPa [185]), is being up regulated, which compensates for the lack of RodZ, restoring cell morphology but not cell viability. This is plausible, in that RodZ is believed to have both a structural role and a role in the segregation of DNA into the daughter cells during replication [193].

Figure 5.15: Images of rodZ mutant strain at 0.1MPa and 30MPa. At 0.1MPa rodZ mutant cells are very round and large, this phenotype is somewhat restored to the parent phenotype at 30MPa. This may be due to the over expression of FtsZ at this pressure which can partly compensate for the loss of RodZ, but not sufficiently to allow growth. The complimented strain does not show the large round morphology at 0.1MPa. Bar = 10µm

5.8 Pressure Killing

The mutants discussed above have been shown to have a pressure sensitive growth phenotype; they are unable to gain biomass at pressures as low as 25MPa. It is not yet established whether there is a link between the physiology of growth at
moderate pressure (30MPa) as studied here, and survival at much greater pressure (600MPa), such as those used in food processing. Understanding the mechanism underlying survival at high pressure is important as this knowledge would allow for longer shelf life of food products and thus greater commercial value. Genes which have been shown to be essential for survival after high pressure processing include \(rpoS\), \(rpoE\), \(dps\), \(trxA/B\), \(hns\), \(nlpIA/B\), \(otsA\), \(katE\), \(oxyR\), \(sodA/B\) and \(soxS\) in O157 [80]. Interestingly there is no overlap between these genes and the genes identified in my work as being essential for growth at 30MPa. To investigate the potential link between growth at pressure and survival after high pressure, I decided to test whether some of the mutants identified in my 30MPa screens also showed a pressure sensitive phenotype at killing pressures.

A typical pressure treatment for sterilizing food stuff is 8min exposure to 600MPa [80]. This results in roughly 0.01% survival for \(E. coli\) strain J1 in stationary phase [80]. It has been shown, in previous work, that mutants which lack the sigma factor gene \(rpoS\) are sensitive to pressure killing, due to the inability to up-regulate stress factors in stationary phase [186]. For this reason I included an \(rpoS\) mutant in my experiments, together with three pressure sensitive mutants (\(priA\), \(holC\) and \(dnaT\)) and the parent strain BW25113.

Since the maximum pressure of the large pressure vessel is only 60MPa, the high pressure microscope cells were used for these pressure killing experiments. The maximum pressure attainable in Cell 2 is 700MPa (see Chapter 3).

Briefly, the mutant strains were cultured at 0.1MPa and 37°C with 200rpm shaking overnight. The resulting stationary phase culture was loaded into the sample chamber of pressure cell 2 and sealed using the solid piston. The pressure cell and piston were loaded into the ram, pressurised to 600MPa and maintained at pressure for 10min. After the pressure was released 10\(\mu l\) of culture was removed and added to 90\(\mu l\) of fresh LB medium, giving a \(10^{-1}\) dilution and serial dilutions to \(10^{-8}\) were made. These dilutions were plated onto LB agar and incubated at 37°C overnight. The next day colonies were counted, and the percentage survival
calculated. All pressure killing experiments were done in triplicate.

Figure 5.16: Survival of several pressure sensitive growth mutants after treatment at sterilizing pressures. After treatment at 600MPa for 10min the survival of the priA mutants is significantly reduced compared to the parent strain. The rpoS mutant also has reduced survival, as expected.

Three of the four mutant strains showed no difference in survival after treatment at 600MPa for 10min when compared to the parent strain. The fourth mutant strain, priA, does show a significantly lower survival after this pressure treatment. However it should be noted that the rpoS mutant should have a much reduced survival under these conditions, which it does not. This may be explained by the difference in genotype of the parent strain BW25113 compared to O157:H7. BW25113 has an additional 13 mutations from the wild-type strain W3110, which is a K12 strain whereas O157:H7 is a significantly different wild-type background with almost 1000 additional genes when compared to W3110.
5.9 Discussion

In order to identify the essential genes for growth at moderate pressure in *E. coli* I used a well characterized library of mutants, the Keio Collection. The reason for choosing this library was two-fold: firstly there are large databases and over 100 publications already produced using this library and secondly all essential genes for “normal” growth have been identified. So in combination this makes for a very powerful tool in identifying genes and gene-products which are essential under specific conditions.

In order to identify genes which are essential for growth a method of quantifying growth was needed: this is no easy matter when dealing with growth in liquid filled high pressure vessels. The sample has to be in a 96-well format, kept separate from the surround liquid and has to have 2 flat windows through which to measure optical density (OD). In the lab of Doug Bartlett a method using 96-well PCR plates and “matt-caps” was used in combination with a metabolic die (phenol red) as an indicator of fermentative growth [169]. In this study growth was observed as a change in colour from red to yellow, however this was not always ideal as it was not quantitative. In order to overcome this I developed a method of sealing flat-bottomed 96-well plates with an optically clear adhesive film. This gave huge scope to measure OD, fluoresce and luminescence in a high throughput manner for the first time at pressure.

After screening the Keio collection at 0.1, 30 and 50MPa an obvious trend was seen: as pressure increased growth decreased for all mutants and parent strains. This indicated that pressure has an effect on the cell as a whole but the lack of correlation between final OD measured at 30MPa and 50MPa suggests that the growth limiting factors at a specific pressure do not become further limiting at an increased pressure; i.e. if pathway A is the limiting factor at 30MPa it is unlikely to be limiting at 50MPa: pathway B now becomes a limiting factor.

In addition to the general effect of pressure on the mutant population
specific mutants were identified which are unable to grow at pressures otherwise permissable to the parent strain. These mutants were considered to lack an essential gene for growth at pressure. Interestingly, but not unexpectedly, no mutations were found which increase pressure resistance.

Those mutants which were identified as lacking genes essential for growth at pressure fall into four groups based on either the function or location of the missing protein, these are: DNA replication and repair, structural proteins, membrane bound proteins and unknown proteins.

The first group, DNA replication and repair, are the most pressure sensitive starting to become essential at \( \sim 25\text{MPa} \). Three of the four mutants in this group are directly involved in the replication of the lagging strand of the DNA replication fork. Where a counterpart for the leading strand exists, as is the case for HolC (HolD), this protein is not essential for growth at pressure. This strongly suggests that there is something specific to the function of the lagging strand of the DNA replication fork which is susceptible to pressure. Even though none of these mutants has been identified as being essential for growth at pressure previously there is precedent for the DNA replication process being pressure sensitive. It was previously shown that an \textit{E. coli} recD mutant is not pressure sensitive unless carrying a pUC18 plasmid \cite{187}. The RecD protein is involved in the initiation of replication of plasmid DNA. The actual mechanism by which DNA replication is pressure sensitive remains unclear: it could be related to the thermodynamics of DNA/RNA polymerisation which excludes solvent from the inner core of the double helix creating an increase in \( \Delta V \) which is unfavourable at increased pressure. The effect may also be due to the weakened protein-DNA/RNA interactions at pressure which are overcome by the presence of the essential proteins or there may be other mechanisms which are less obvious. It is interesting to note that the lagging strand of the DNA replication fork is thought to move; the fork having three prongs, a stationary one for the leading strand and two moving strands repositioning along the lagging strand to create new Okazaki
fragments. The primosome is also thought to attach and detach along the lagging strand during DNA replication.

The \textit{nus}B mutant is less pressure sensitive than the other DNA replication/repair mutants and this is likely due to it’s role as a regulator and transcription terminator rather than its role in replicating DNA. The proteins which are regulated by NusB activity have not been fully explored but it is known to be involved in cold shock adaptation. Cold shock genes have been shown to be up-regulated in response to high pressure treatment in \textit{E. coli} and it may be through the loss of this activity that NusB becomes essential[185].

The cell shape mutants which are essential for growth remain a somewhat mysterious as there is only one paper on each protein. It is however known that both RodZ and DedD interact with FtsZ, a protein which has been shown to depolymerize at pressures $\sim40\text{MPa}$ in vitro [165]. RodZ is known to attach to MreB which acts as the bacterial cytoskeleton on which FtsZ binds. During the polymerisation of FtsZ the MreB cytoskeleton is pulled closer together until the two daughter cells are almost separate. It is at this stage that DedD attaches to the septal ring and initiates the separation of the two daughter cells. DedD is not essential in this last stage under normal growth conditions as mechanical separation has been observed in this mutant.

The data presented in this thesis suggests that the mechanism through which these two genes become essential is linked to their interactions with FtsZ. This is supported by the face that \textit{ded}D mutants are hyperfilamented when grown at 30MPa. The \textit{rod}Z mutants form rod shaped cells at 30MPa may be explained by the over expression of FtsZ seen in microarray data for \textit{E. coli} grown at 30MPa [185]. I propose that at pressure polymerisation of FtsZ is hindered but this can be compensated for by over expression of FtsZ. However in the absence of RodZ, FtsZ cannot bind as efficiently to the MreB cytoskeleton and effectively cells cannot replicate. It may be that RodZ helps FtsZ to polymerise so in its absence the effects of pressure are greater and cells cannot replicate. DedD may play a
similar role in stabilizing the FtsZ ring at pressure so that its absence reduces the pressure tolerance for FtsZ polymerisation. Actually testing this hypothesis would require significantly more research.

The membrane bound proteins can be further divided into two groups: firstly those importing or exporting proteins and those importing or exporting protons. The membrane bound proton-import protein, AtpG, is a key factor in the production of ATP, the major energy currency of the cell. Under atmospheric pressure the membrane has a fluid liquid crystal structure allowing the free rotation of the F1-F0 complex, of which AtpG is a part, and the production of ATP. In the absence of AtpG this process is still able to occur at atmospheric pressure but with the phase transition of the cell membrane the F1-F0 complex may be less able to move and may even disassociate completely. The membrane proteins which are involved in protein transport add another layer of complexity to the story since they may, in themselves, not be essential for growth at pressure, but it is also possible that through the loss of these pore-forming proteins other, essential proteins are not able to gain access to the periplasm or cytoplasm (TatC exports to the periplasm and TolQB import into the cytoplasm). During the initial, non-quantitative, screen I identified envC as being essential for growth at 30MPa but during subsequent repeats this mutants sensitivity was not reliable and it was therefore discounted from further study. However the EnvC protein is exported into the periplasm via the Tat system, which tentatively supports the hypothesis that TatC is not, in itself, essential for growth at pressure but rather a protein which it transports. Countering this argument is the fact that none of the other proteins which have been identified in this study are exported by the Tat system or associated with the Tol system.

While none of these mutants have precisely been identified as being involved in pressure adaption or sensitivity there is a correlation with other data for growth at high pressure. For instance the \textit{P. profundum} SS9 genome has many genes whose products are involved in transport across the membrane \cite{41}. The microarray
data for *P. profundum* SS9 also indicates that transport genes are up-regulated at pressure [108]. There is also microarray data for *E. coli* which shows a similar pattern [185].

The remaining mutants are more difficult to analyse as their function is either hypothetical, inferred from other organisms or is totally unknown.

A common theme for these mutants is that their deleted gene products all form part of a larger protein complex. Could this be key to the mode of action pressure has on them? It is known, as discussed earlier, that pressure affects protein-protein complexes as well as DNA-protein complexes. This is thought to come about due to the exclusion of water from the inter-molecular binding surface of the molecule via hydrophobic interactions, which on turn causes an increase in molecular volume and is disfavored at pressure. If these protein complexes come apart they are no longer able to function.

Had time allowed I would have liked to have carried this project much further. In particular I would P1 transduce the mutation into a “clean” background and ensure that the pressure sensitive phenotype was retained. BW25113 has an additional 13 mutations compared to the wild-type strain W3110, and it could be the synergistic effect of these mutations which cause the pressure sensitive phenotype seen. This is unlikely however as the complementation of the pressure sensitive phenotype is seen only when re-introducing the 14th deleted gene. Furthermore I would have liked to remove the kanamycin resistance cassette as this may have undesired down-stream effects on the genes in an operon.

A more interesting, but much larger experiment would be to over express proteins and screen for the ability to grow at increased pressures. This could be done with the ASKA collection at 45MPa; a pressure which would reduce growth for all cultures except those with increased pressure resistance due to over expression of specific proteins. Such a study would be very interesting and complimentary to the experiments in this thesis.
Chapter 6

Conclusions

The world is dominated by microbes: there is no environmental niche which is not colonized by these organisms. Among the environments colonized by microbes is the deep sea, one of the largest environments on Earth. In order to understand how bacteria are adapted to the deep sea model organisms are needed; these are organisms for which a head of knowledge has already been built up and for which new tools and techniques are being developed. The deep sea model microbe is *Photobacterium profundum* SS9. This bacterium has provided numerous insights into the genetic and physiological adaptations needed to survive in the deep sea.

The majority of the work carried out on *P. profundum* SS9 has been in the adaptation to cold temperatures and high pressures as these are the two dominant features of the deep sea. Another, less studied, feature is the high, and sometimes varying salt conditions in the deep sea. It was this combination of high pressure and osmotic pressure which I chose to study in my PhD.

The first obstacle in studying the adaptation of bacteria at high pressure and varying osmotic pressure was the ability to grow bacteria under these conditions. I found the bags and bulbs methods of culturing at pressure cumbersome and subject to contamination from the pressurising medium. To get around this I developed a method of sealing 96-well plates which allowed the cultures within
to remain isolated and able to be brought in and out of the pressure vessel with ease.

Using these modified 96-well plates I was able to obtain high-throughput data on the growth of *P. profundum* SS9 and 3TCK at different pressures and salt concentrations. This experiment resulted in a very complex set of data, which generated more many more questions than it provided answers. But I feel this is the way of good science; the further we delve the more we realize we do not know.

As proof of the flexibility of this high-throughput method I then went on to screen a library of over 3900 single gene deletion mutants of *E. coli* for the ability to grow at pressure. To the best of my knowledge, this is the first time this type of experiment has been carried out. This experiment highlighted a set of essential genes for growth at permissive pressures none of which had previously been identified although the physiological functions of the genes had in many cases previously implicated in pressure sensitivity.

Further to the development of a high-throughput growth method I helped develop an optical pressure cell for imaging bacteria at pressure. This system addresses many of the problems in imaging bacteria at pressure, such as depressurization artifacts and the poor resolution of other pressure cell designs. However there are still biological limitations which need to be addressed, such as the inability of GFP to fold correctly under anaerobic conditions. While there are developments in this direction, such as LOV-proteins, would have been beyond the scope of this project.

Not all the aspects of my PhD have met with success. I believe that a critical decision at the start of my PhD, where I chose HPLC over NMR, lead to many of the problems in this part of my project. Had I chosen to use NMR the cost would have been higher and data analysis more difficult, but neither of these obstacles would have been impossible to over come. The experiment could have been re-designed to reduce costs and with the right software and training data
analysis would have become easier over time. However I chose to use HPLC, not at the time appreciating the complexity of the technique and the effects varying salt would have on the retention times of each sample. I do believe that had time allowed a suitable HPLC protocol could have been developed. The extracts I produced in this experiment remain in the freezer and it may be possible that at a future data.

The other area which I struggled in this PhD was the complementation of the TW30 mutant strain. While I set out to determine whether it was the toxR or toxS deletion which caused the different growth phenotype observed in TW30 as compared to SS9, by creating independent clones of my own, I should have spoken to Doug Bartlett and enquired about the complimented strain his lab had already produced. Had I done this I could have acquired this strain sooner and been able to carry out the experiments required to prove the link between ToxR and pressure adaptation. I would have also dearly loved to have done microarray studies of the TW30 and SS9 strains and compared gene expression at 0.1MPa and 30MPa. This would have provided a fascinating insight into the role of ToxR in pressure adaptation in *P. profundum*.

During my PhD I set about to answer some questions as to how osmotic and hydrostatic pressure affect bacteria. While not every avenue of investigation was successful I believe I have taken some significant steps in answering these questions.
Publications


* Equal contribution

S. Lucas Black, F. Bruce Ward and Rosalind J. Allen. Stress intensity-dependent genome-wide screening reveals a hierarchy of essential genes for *Escherichia coli* at high pressure *In preparation*
Bibliography


