THE EFFECT OF MANUKA HONEY ON THE CELL CYCLE OF MRSA

Thesis submitted in candidature for the degree of

DOCTOR OF PHILOSOPHY

By

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Posters presented:


**Oral Presentations**


15th May 2006 “The effect of Manuka honey on the cell cycle of MRSA” UWIC, Cardiff School Health Sciences Annual Postgraduate and KTP Colloquium, Gregynog Hall, Newtown, Powys
Articles

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Abstract

Preliminary studies have shown that manuka honey affects the cell cycle of MRSA by impeding cell division, but mode of action was unknown. Cell division depends on the formation of septa and cleavage of peptidoglycan at cytokinesis. This study investigated how manuka honey might alter the cell cycle of EMRSA-15. Physiological and chemical changes in the bacteria exposed to manuka honey were determined using time to kill studies, confocal and electron microscopy. Data indicated that honey had a bactericidal effect on MRSA, inhibiting the cell cycle cytokinesis. Increased septum formation was noted in honey treated cells by transmission electron microscopy. Cell division components including FtsZ and Endo-B-N-Acetylglucosaminidase were investigated using cell wall turbidity assays, zymography, immunofluorescence and immuno gold labelling. Manuka honey treated MRSA cells showed a marked reduction in hydrolase activity after 12 hours compared to untreated cells. The immunofluorescence indicated an initial increase in FtsZ production followed by a significant decrease by 24 hours. PCR of FtsZ showed a 10% increase in production after 1 and 4 hours. Localization by gold labelling gave inconclusive results. Immunofluorescence of Endo-B-N-Acetylglucosaminidase showed a decrease in the amount of enzyme over 24 hours and localization by gold labelling indicated altered distribution of this enzyme. PCR showed no significant difference in expression. 2-D electrophoresis showed a differing proteomic profile between control cells and those treated with honey, with a potential target protein being identified. Methylglyoxal (an antibacterial component of manuka honey) was investigated after a report named this as potentially the active component of manuka honey. Results showed it has an effect but is not wholly responsible for the effects induced by manuka honey. It was concluded that increased numbers of cells with septa were formed and alteration in production of proteins and enzymes resulted in MRSA cells exposed to bactericidal concentrations of manuka honey. The work was also carried out with artificial honey controls, indicating that effects seen were not due to sugar content within honey or methylglyoxal content.
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List of Abbreviations:

Agr – Accessory gene regulator
Atl – Autolysin
BDMA – Benzylidimethylamine
BSAC – British Society of Antimicrobial Chemotherapy
CLSI – Clinical and Laboratory Standards Institute
DDSA – Dodecenyl succinic anhydride
EMRSA-15 – Epidemic meticillin resistant *Staphylococcus aureus* -15
FACS – Fluorescence-activated cell sorting
FtsZ – Filamentous temperature sensitive protein Z
Gcp – Glycoprotease
GFP – Green fluorescent protein
H₂O – Water
IEF – Isoelectric focusing
IPG – Immobilised pH gradient
Lyt – Lysostaphin type autolysin
MALDITOF – Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MBC – Minimum bactericidal concentration
MIC – Minimum inhibitory concentration
Mg/ml – Milligram per millilitre
MGO – Methylglyoxal
MRSA – Meticillin resistant *Staphylococcus aureus*
NaCl – Sodium chloride
NB – Nutrient broth
**Rat** – Regulator of autolytic activity

**Rpm** – Revolutions per minute

**Sar** – Staphylococcal accessory regulator

**SDS** - Sodium dodecyl sulphate

**SEM** – Scanning electron microscopy

**TEM** - Transmission electron microscopy

**TSB** – Tryptic soy broth

**TVC** – Total viable count

**µl** – Microlitre

**UMF** – Unique manuka factor

**VBNC** – Viable but non culturable count

**VISA** – Vancomycin intermediate *Staphylococcus aureus*

**2D** – Two dimension
1. Introduction

Since ancient times honey has been valued as having medicinal properties. Its use after the advent of antibiotics was dramatically reduced, but since the occurrence of antibiotic resistance certain honeys have been re-evaluated as antibacterial agents. Manuka honey is a honey that has a phytochemical component distinct from many other honeys that seems to give it a unique antibacterial effect. However the mode of action of this natural product is unknown, leading to reluctance on the part of some medical practitioners to use it.

It has been noted that manuka honey has an antibacterial effect on many species of wound infecting bacteria including meticillin resistant Staphylococcus aureus (MRSA) (Molan 1992; Blair & Carter, 2005). While the antibacterial effect of this honey is well recognized, its mode of action has not been explained. In particular the bactericidal effect elicited on MRSA which explains increasing reports of its eradication from wounds (Dunford et al, 2001; Kwakman et al, 2005; White, Cooper and Molan, 2005; Simon et al, 2006) has not been investigated.

1.1 Honey and its history of healing

Bees have been in existence for over one hundred and twenty five million years and they have been successful from an evolutionary perspective in that they can exploit virtually all habitats on earth. Their success is partly due to the chemistry and application of the many of their products such as: beeswax, venom, propolis, royal jelly and honey (Bankova, 2005). All of these products have been shown to possess varying nutritional and medicinal
properties that have meant that man and bees have established a symbiotic relationship (Meda et al, 2004).

Honey has been used by humans since ancient times as both a dietary source and sweetener, and until recent times it was also highly regarded as a traditional medicinal treatment for many ailments (Crane, 1999; Willix et al, 1992). Honeys have been extensively used as a topical therapeutic agent in clinical trials on abscesses, ulcers and burns (Molan, 2001; Jull et al, 2008; Brady 2008). A range of positive benefits have been suggested when used to treat these conditions, including reduction of inflammation, pain reduction, reduction of odour, debridment of necrotic tissue and promotion of granulation and epithelization (Tonks et al, 2001), but the design of clinical trials has been criticized (Jull et al, 2008b; Bardy et al, 2008).

1.1.2 Ancient Times

Recognition of honey as an important food source and valuable product has been seen from as far back as ten thousand years ago. Cave paintings from around the world have shown that honey collection figured in the lives of early man (Crane, 1975; Jones, 2001; Blair, 2009). It would seem that honey was regarded as a prime tool in many different parts of the world such as Britain, Asia and Egypt, and many communities seem to have come to the conclusion that honey was of value independently of one another. It was used not only as a food source but in religious ceremonies and as a medicine.

The earliest civilization to have recorded beekeeping as a profession were the Egyptians, possibly as early as 5000 BC, and illustrations in temples showed keepers using smoke to
calm bees (Crane, 1999). Egyptians were also known to use honey extensively as a medicine as recorded in the Edwin Smith Surgical Papyrus (Atta, 1999; Feldman and Goodrich, 1999; Ovington, 2002).

The Romans regarded it highly and used honey and propolis, another substance produced by bees, as one of their most common prescriptions (Crane, 1975; Dealy, 2006; Jones, 2001). As far back as Aristotle (384-322 BC) and Dioscorides (c.50 AD) different honeys were recognised as having differing properties; pale honey being recommended for eye wounds by Aristotle and Attican pale yellow honey was recommended for ulcers by Dioscorides.

1.1.3 Middle Ages to Modern Day

After the fall of the Roman Empire, honey continued to be utilized across the world in places such as India, Greece and Egypt, for many uses including; cleaning and disinfecting wounds, mouthwash, aphrodisiac and as an eye balm (Jones, 2001; Crane, 1975). Honey became a valued product in Britain and as sugar became more easily available honey was viewed as a luxury item. The price and availability of honey depended on its production and ease of distribution in countries across Europe (Crane, 1975). However, by the 1900s honey was being commercially produced and harvested across the world.

There has been a recent interest in the use of honey as an antibacterial agent due to emergence of antibiotic resistance both in hospitals and in the community. The use of honey as a therapeutic product is closely regulated by the Medicine Act of 1968, currently honey based wound products are licensed as devices not as medicines (Cooper, 2005). The first available honey based wound product in the United Kingdom was a manuka honey
containing device made available on 1st March 2004. There are currently several medical products available on the market that use honey as their active ingredient including dressings from Advancis medical, Mesitran and Activon. As research has developed it has become clear that some honeys have antibacterial properties from a phytochemical component not seen in the majority of honeys. It has been suggested that this phytochemical component may be methylglyoxal previously known as the unique manuka factor and is found in honeys derived from nectar collected from the flowers of Leptospermum species. The selection of honey for use in wound healing is an important decision and is based on the properties of the honey.

1.2 Characteristics of honey

Honey is a substance made from the processing of nectar collected from mainly the nectarines of flowers by bees, it can also be made from collecting honey dew or sap from some species of flowers, depending on the location and species of the bees. Nectar consists mainly of an aqueous solution of sugars and small amounts of ash, minerals, aromatic substances and vitamins. Bees usually collect at the peak time of nectar production, and can collect up to 90% of their bodyweight in nectar, carrying it in the nectar sack in which it begins to undergo some transformation. Nectar is returned to the hive and passed to house worker bees which put the nectar through a series of regurgitations and take it to the honeycomb. The honey is ripened and changed by enzymes already present in the raw material collected and also by enzymes added from the secretions of the bees themselves while water content is reduced (Crane, 1975; White et al, 1963).
1.2.1 Physical

Honey is a saturated sugar solution and therefore has an osmolarity high enough to inhibit bacterial growth (Molan, 2001). The physical properties of honey are useful not only in providing a barrier to infection when applied to a wound but also as a means of distinguishing between honeys. One of the most obvious physical characteristics of honey is colour. The colours can range from almost black through amber to white, and this can be helpful in the identification of floral source of the original nectar of differing honeys.

Honey also has a range of viscosities; these can be altered depending on the temperature at which they are measured. The colour and consistency of honey is not only affected by the source of flower from which the nectar was collected but is also affected by variables such as weather and climatic changes (Molan, 2006).

1.2.2 Chemical

Honeys are very complex substances consisting of 200 – 600 substances including sugars, vitamins, minerals, lipids and acids (Bogdanov, 2004). These many components are normally contained in a similar composition ratio in different honeys (Figure 1, Table 1).

The complex chemical composition of honey confers three recognisable properties that can cause an antibacterial effect before the unique manuka factor is taken into consideration. One is the osmotic effect caused by the supersaturated nature of honey, which leaves little free water available for pathogenic growth. The free water that is found in honey is known as the water activity (Aw) and in most honeys it has a value of between 0.562 to 0.62, Staphylococcus aureus need a free water value of above 0.897 to able to grow freely (Cooper et al, 1999). The acidic pH of honey (normally pH 3.2 – 4.5) also limits or inhibits
the growth of many organisms. Animal pathogens generally need a pH of 7.2 – 7.4 for optimum growth.

![Pie chart of major nutrients in honey](image)

**Figure 1: The major components of honey (White, 1979).**

<table>
<thead>
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<th>Average amount per 100 g</th>
<th>Minerals</th>
<th>Average amount per 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>0.04 mg</td>
<td>Calcium</td>
<td>6.00 mg</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.12 mg</td>
<td>Phosphorus</td>
<td>4.00 mg</td>
</tr>
<tr>
<td>Pantothenic</td>
<td>0.07 mg</td>
<td>Sodium</td>
<td>4.00 mg</td>
</tr>
<tr>
<td>Vitamin B-6</td>
<td>0.02 mg</td>
<td>Potassium</td>
<td>52.0 mg</td>
</tr>
<tr>
<td>Folate</td>
<td>2.00 mg</td>
<td>Iron</td>
<td>0.42 mg</td>
</tr>
<tr>
<td>Vitamin</td>
<td>0.50 mg</td>
<td>Zinc</td>
<td>0.22 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Magnesium</td>
<td>2.00 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Selenium</td>
<td>0.80 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Copper</td>
<td>0.04 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maganese</td>
<td>0.08 mg</td>
</tr>
</tbody>
</table>

**Table 1: Vitamins and minerals found in honey per 100 g (White, 1979).**

The ability of honeys to produce hydrogen peroxide through the glucose oxidase enzyme pathways is considered to be the most potent antibacterial factor in honeys not containing
the unique manuka factor (Ahmed et al, 2003; Bang et al, 2003; White et al, 1963). The glucose oxidase enzyme is secreted from the hypopharyngeal gland of the bee into the nectar to assist in the formation of honey from the nectar. The hydrogen peroxide and acidity found in honey are produced by the reaction and serve in nature to preserve the honey (White et al, 1963).

\[
\text{glucose} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2
\]

The use of hydrogen peroxide as an antimicrobial agent is effective at high concentration but can cause tissue and cellular damage due to the release of free radicals (Bang et al, 2003). This ability of hydrogen peroxide to cause cytotoxicity to tissues at millimolar concentrations means use of hydrogen peroxide alone as an antibacterial for clearing wounds has been discouraged (Lineaweaver et al, 1985). Honey however releases low levels of hydrogen peroxide over time making it safer to use, also leading to the study of honeys that have activity additional to or instead of its peroxide activity.

### 1.2.3 Manuka

Manuka honey is honey produced by bees feeding on the pollen contained within the flowers of the plant *Leptospermum scoparium* (manuka bush). This is a shrub found widely in New Zealand and Australia; it grows in many locations for a number of months and can have white, pink or red flowers (Figure 2).
Figure 2: *Leptospermum scoparium* (manuka bush) White and red varieties of the manuka bush from New Zealand (image taken from© (2008) Paul Ashford, www.NZplantpics.com).
Only honey made from bees feeding exclusively on this plant can be said to be manuka honey and it is thought that it is something from this plant that gives the manuka honey its unique activity. Manuka honeys are even graded with a UMF (unique manuka factor) which gives an estimate of the potency of the honey compared to phenol. The unique manuka factor is not affected by the catalase enzyme found in body tissue that honey will come into contact with if placed in a wound nor is it affected by heat or light. This makes it unique because the other factors causing antibacterial activity in honey can be destroyed by enzymes or heat.

Over the years honey has been fractionated into various components as a more complete understanding of the composition of honey and the potentially antibacterial parts has been sought (Weston, 1998; Suarez-Luque, 2002). Despite the many investigations into the organic, inorganic phases and active ingredients in honey, manuka honey is still used medically as a whole honey, because the active components are still largely unknown. Recently it has been discovered that a proportion of the non peroxide antibacterial activity found in manuka honey could produced by methylglyoxal (Adams, 2008; Mavric, 2008). This is a highly reactive precursor of advanced glycation endproducts. It has been detected in manuka honeys using HPLC with UV detection and also by o-phenylenediamine derivatisation. Both methods showed concentrations of methylglyoxal in the honey between around 38 – 828 mg/kg, which correlated with the non peroxide antibacterial activity of the honeys (Adams, 2008; Mavric, 2008). Although these two studies have shown activity from this fraction of manuka honey it is unlikely that this will alter the products currently on the market until further extensive studies are done showing that this chemical is indeed the only part of honey causing the beneficial antibacterial and wound healing effects seen in previous studies.
**1.3 Meticillin resistant *Staphylococcus aureus***

*Staphylococcus aureus* was first isolated in the 1880’s (Ogston, 1886; Lyell, 1989) and is a Gram positive bacterium that is found as an intermittently commensal organism nasally in up to 30% of the population, it can also be found in other sites on the body including the groin and gastrointestinal tract (Gordon and Lowy, 2008).

The organism can also act as a pathogen of humans, possessing many virulence factors that help it to evade the immune system and cause clinical symptoms (Table 2).

<table>
<thead>
<tr>
<th>Virulence Factor Type</th>
<th>Selected Factors</th>
<th>Associated Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Involved in attachment</td>
<td>Clumping factors, fibronectin-binding proteins, collagen and bone sialoprotein-binding protein,</td>
<td>Endocarditis, osteomyelitis, septic arthritis, prosthetic device infection.</td>
</tr>
<tr>
<td>Involved in persistence</td>
<td>Polysaccharide intracellular adhesion, small colony variants and intracellular persistence</td>
<td>Relapsing infection and infections as described above.</td>
</tr>
<tr>
<td>Evading immune system</td>
<td>Leukocidins, capsular polysaccharides, protein A and phenol soluble modulins</td>
<td>Invasive skin infections, necrotizing pneumonia, abscesses</td>
</tr>
<tr>
<td>Tissue penetration</td>
<td>Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C and metalloproteases</td>
<td>Metastatic infection, tissue destruction</td>
</tr>
<tr>
<td>Involved in toxin mediated disease or sepsis</td>
<td>Enterotoxins, toxic shock syndrome toxin 1, exfoliative toxins A and B, α toxin, lipoteichoic acid and peptidoglycan</td>
<td>Food poisoning, toxic shock syndrome, scalded skin syndrome, bullous impetigo, sepsis syndrome</td>
</tr>
<tr>
<td>Poorly defined role in virulence</td>
<td>Coagulase, bacteriocin and arginine catabolic mobile element</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Table 2: *Staphylococcus aureus* virulence factors.** Some of the selected factors have more than one role in staphylococcal virulence (Table adapted from Gordon and Lowy, 2008).
Meticillin resistant *Staphylococcus aureus* is an antibiotic resistant strain of the most commonly isolated wound pathogen (Dancer, 2008); it emerged in the 1960s only a year after meticillin was introduced and since then finding alternative ways of eradicating it has become an important area of research (Gibbons, 2008). The importance of finding novel ways of eradicating MRSA from both nosocomial and community acquired infections can be readily understood when it is realized that the mortality rates for staphylococcal bacteraemia were around 80% before the introduction of antibiotics (Dancer, 2008). The rates of mortality dropped after penicillin became widely available in the 1940s but have gradually increased since the problem of resistance emerged (Kirby, 1944) (Figure 3).

**Mortality rates of Staphylococcal bacteraemia**

![Graph showing mortality rates of Staphylococcal bacteraemia over time](image)

**Figure 3:** Human Mortality rates from staphylococcal bacteraemia over time. MSSA – Methicillin-sensitive *Staphylococcus aureus*, MRSA – Methicillin-resistant *Staphylococcus aureus*, VISA – Vancomycin-intermediate *Staphylococcus aureus*. Graph adapted from Dancer, 2008.
Originally strains of *S. aureus* were susceptible to β-lactams (penicillins and cephalosporins) and resistance was generally found only in patients in close contact with hospitals. More recently resistance to glycopeptides has emerged and meticillin-resistant *S. aureus* (MRSA) has been isolated from individuals who have had no contact with hospitals (community acquired MRSA) (Gosbell, 2004).

Community acquired MRSA (CA-MRSA) has been isolated worldwide and has generally been found to be more virulent than hospital acquired MRSA due to the virulence factors found to be present (Deurenburg and Stobberingh, 2008). Initially CA-MRSA was characterized by the presence of Pantone-Valentine Leukocidin, a bicomponent cytotoxin associated with skin problems and necrotizing pneumonia (Boubaker et al, 2004). However, the number of hospital and community acquired infections has risen over the years and the distinction between hospital acquired and community acquired MRSA has become less clear (Loffler and MacDougall, 2007; Lowey, 1998).

### 1.3.1 Cell Structure

The basic structure of *Staphylococcus aureus* is a simple one consisting of three cellular compartments the cytosol, cytoplasmic membrane and the cell wall. The wall of bacteria is a relatively complicated structure that as yet is still not accurately defined (Rice and Bayles, 2008). The cell wall is a three dimensional structure that encases the rest of the cell determining structure and shape, preventing osmotic lysis under hypotonic conditions and providing a scaffold for cell proteins (Figure 4). The cell wall of Gram positive cells such as *S. aureus* is composed of peptidoglycan in a layer of 20 – 40 nm thickness. The chemical structure of peptidoglycan was initially determined in the 1950’s when it was shown that within the basic structure of the cell wall there were many other components including
teichoic, teichuronic and muramic acids, as well as carbohydrates and polyphosphates (Navarre and Schneewind, 1999).

Although the peptidoglycan structure within different bacteria may differ chemically depending on the bridging between polymer strands by amino acids and/or proteins, it has been shown that all are produced by a common sequence. Initially UDP-GlcNAc (N-acetylg glucosamine) is synthesized from fructose-6-phosphate by 4 enzyme reactions, then UDP-MurNAc-pentapeptide (N-acetylmuramic acid) is formed from UDP-GlcNAc via MurA and Mur B enzymes within the cytoplasm. This disaccharide-peptide precursor is then translocated to the external face of the plasma membrane by a lipid carrier, where it is attached to a growing peptidoglycan chain via glycosyl transfer and transpeptidation reaction, forming growing peptidoglycan chains (Barretau et al, 2008; Bouhss et al, 2008).
As peptidoglycan has a role in maintaining bacterial shape, so the other components of the wall have specific roles. It is thought that teichoic acids (a family of carbohydrates) which can make up to as much as 50% of the wall are involved in maintaining a suitable surface charge across the cell. It is important that this surface charge is maintained as it can affect many things from resistance to antibiotics to the ability of bacteria to adhere to surfaces. It can also affect murein (peptidoglycan) hydrolase activity, which is important in cell growth and division (Rice and Bayles, 2008). The peptidoglycan forms a polyanionic gel structure around the cell and has extensive functions from maintaining flow of nutrients, ions and proteins to and from the cytoplasmic membrane, and most importantly protecting the cell from turgor pressure and if this wall is breached by mutations, antibiotic action or enzyme damage it can lead to cell lysis and death (Vollmer et al, 2008).
1.3.2 Cell Cycle

The cell cycle is a complex and organized series of events in which increase in cell size, DNA replication and segregation, followed by cell division leads to the formation of daughter cells which each receive a complete copy of the original genome (Amick and Brun, 2001).

The architecture of the cell is composed of many elements including homologues of eukaryotic actin, tubulin and intermediate filaments which provide a flexible scaffold that helps to maintain cell shape, facilitate DNA segregation and promote cell division. These components are, therefore, potential antimicrobial targets (Table 3) (Moller-Jensen and Lowe, 2005).

Most of the investigative research work for the characterization of these systems has been done in *Escherichia coli*. This demonstrates that MinC is a division inhibitor that is targeted to MinD within the plasma membrane and jointly (MinCD) targeted to a septal component which is prevented from forming in the wrong place by joint oscillation of itself and MinE (Zhou and Luktenhaus, 2005). The DivIVA complex which recruits MinCD to cell poles in other cells is present in *S. aureus* however there is no MinCD complex. Whereas the position of cell division in rod shaped bacteria does not vary, the situation is different in cells of *S. aureus* because division plane changes in three consecutive perpendicular orientations in successive division cycles (Pinho and Errington, 2004).
### Bacterial cytoskeleton components

<table>
<thead>
<tr>
<th>Name</th>
<th>Homology</th>
<th>Function</th>
<th>Intracellular localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FtsZ</td>
<td>Tubulin</td>
<td>Primary cell division protein</td>
<td>Dynamic Z-ring placement</td>
</tr>
<tr>
<td>FtsA</td>
<td>Actin</td>
<td>Stabilizes Z ring, recruitment of division proteins</td>
<td>Z-ring</td>
</tr>
<tr>
<td>ZipA</td>
<td></td>
<td>Stabilizes Z ring, recruitment of division proteins</td>
<td>Z-ring</td>
</tr>
<tr>
<td>ZapA</td>
<td></td>
<td>Cross linking FtsZ filaments</td>
<td>Z-ring</td>
</tr>
<tr>
<td>SulA</td>
<td></td>
<td>Stress inhibitor of FtsZ polymer assembly</td>
<td>Z-ring</td>
</tr>
<tr>
<td>EzrA</td>
<td></td>
<td>Inhibitor of FtsZ polymerization, division site placement</td>
<td>Membrane associated</td>
</tr>
<tr>
<td>MinC</td>
<td></td>
<td>Inhibitor of FtsZ polymerization, division site placement</td>
<td>Oscillating/Polar</td>
</tr>
<tr>
<td>MinD</td>
<td>Nitrogenase iron protein</td>
<td>Binds MinC to determine division site placement</td>
<td>Oscillating/Polar</td>
</tr>
<tr>
<td>MinE</td>
<td>MinC/D regulator</td>
<td></td>
<td>Oscillating</td>
</tr>
<tr>
<td>MreB/Mbl</td>
<td>Actin</td>
<td>Chromosome segregation</td>
<td>Helical, dynamic</td>
</tr>
<tr>
<td>ParM</td>
<td>Actin</td>
<td>Plasmid segregation</td>
<td>Dynamic filaments</td>
</tr>
<tr>
<td>SetB</td>
<td></td>
<td>MreB associated, chromosome segregation</td>
<td>Helical, membrane localised</td>
</tr>
</tbody>
</table>

**Table 3: Cytoskeleton components of bacterial cells.** FtsZ is studied in detail in chapter 5, not all of these division proteins are found in *S. aureus* (Adapted from Moller-Jensen and Lowe, 2005). Those components in bold are found in *S. aureus.*
Cell division assembles these cell division proteins in a hierarchy, in many organisms the placement of the Z ring is controlled by nucleoid occlusion and by the Min system; MinC, MinD and the earliest gene product identified as being involved in cell division is FtsZ, this protein assembles into a ring at mid cell (Blaauwen et al., 1999). The Z ring is present in nearly all bacteria; formation of the Z ring occurs after the separation of the newly synthesized chromosomes into two discrete copies. The Z ring is formed by polymerisation of FtsZ at mid cell which then acts as a framework for at least 12 other division proteins to recruit to in a mostly linear order (Corbin et al., 2004; Pichoff and Lutkenhaus, 2007) (Figure 5). The cell then divides via invagination of the leading edge of the septum and the action of peptidoglycan hydrolases (murein hydrolase) on the wall between the daughter cells (Priyadarshini et al., 2007).
Figure 5: Recruitment of proteins to the Z ring in *S. aureus*. The FtsZ ring forms across mid cell, and the remaining proteins are then recruited in linear order (top to bottom on diagram). Once all the proteins are recruited, constriction of the Z ring occurs leading to formation of two daughter cells. Adapted from Arends and Wiess, (2004).

1.3.3 Murein Hydrolases:

Murein (peptidoglycan) hydrolases are bacterial enzymes ubiquitous among bacteria that digest the bacterial cell wall peptidoglycan of the bacteria that produce them (Mani *et al*,...
1993). Lytic enzymes such as the murein hydrolases were first studied and reported in *Staphylococcus aureus* by Welsch and Salmon, (1950) and Mitchell and Moyles, (1957) (Singer *et al*, 1972). Further studies have shown a variety of hydrolases, both intracellular and extracellular, with lytic functions.

The structure and biosynthesis of staphylococcal peptidoglycan has been extensively investigated (Schleifer and Kandler, 1972; Rogers *et al*, 1980) and it has been revealed that the glycan chains are relatively short and are highly cross linked with peptide chains. There is a high degree of cross linking in the peptidoglycan sub units made possible by the long and flexible pentaglycine interpeptide bridges that span between peptides that normally would be at a distance too far apart: over 95% of the peptidoglycan subunits are cross-linked.

The peptidoglycan structure of *S. aureus* that the peptidoglycan hydrolases act on is a highly modified one compared to other bacteria such as *E. coli* (Rice and Bayles, 2008; Vollmer, 2008b). There are almost no free carboxyl groups as the alpha-carboxyl group of D-glutamic acid is amidated, also around 50% of the muramic residues are 4-N, 6-O- diacetyl-derivatized. This structure means that the peptidoglycan is resistant to lysozyme from humans and egg white. It is a highly dynamic structure, while also being strong and expandable as the cell grows, reshaping as the cells differentiate and divide.

**1.3.4 Antibiotic Resistance**

The mechanism of bacterial resistance to each antimicrobial agent has evolved over time and is dependant on the type of drug (Table 4). In penicillin resistant *S. aureus* the production of β lactamases, enzymes that hydrolyse the amide bond of the β lactam ring,
inactivates the antibiotic (Gardam, 2000). The mechanism of resistance to meticillin is
different to that of penicillin, despite the two drugs both being β-lactam antibiotics (drugs
that target the cell wall). The meticillin resistance seen in MRSA is caused by the
acquisition of a gene that codes for the production of penicillin binding protein (PBP2a)
that is altered from the original PBP, causing it to have a reduced affinity for β-lactam
antibiotics (Lowy, 2003).

1.3.5 New antimicrobials

Bacteria have shown great versatility in developing resistance to drugs and overcoming
antimicrobial challenges, this in turn complicates treatment of bacterial infections. There
are many types of resistance now recognised and lateral gene transfer can allow these
resistances to be passed between species and the problem of single and multiple drug
resistance in bacteria is spreading (Table 4) (Tenover, 2006).

Development of inhibitors to known resistance gene products and modification of drugs
already in use may provide new antibacterial therapy in the short term but it has become
increasingly obvious that new drug targets and development of novel inhibitors is necessary
to overcome the current problems of resistant bacteria (White and Kell, 2004).
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistance Gene</th>
<th>Gene product</th>
<th>Mechanism</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactam</td>
<td><em>BlaZ</em></td>
<td>B-lactamase</td>
<td>Hydrolysis of cyclic amide bond in beta-lactam ring. Lower affinity for PBP</td>
<td>Pl-Tn</td>
</tr>
<tr>
<td>Eg. penicillin</td>
<td><em>MecA</em></td>
<td>PBP2a</td>
<td></td>
<td>C-SCCmec</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td><em>vanA - G</em></td>
<td>Altered peptidoglycan. D-Ala-D-Lac</td>
<td>Vancomycin trapped in cell wall. Lower affinity for vancomycin</td>
<td>C</td>
</tr>
<tr>
<td>Eg. vancomycin</td>
<td></td>
<td></td>
<td></td>
<td>Pl-Tn</td>
</tr>
<tr>
<td>Quinolones</td>
<td><em>ParC</em></td>
<td>ParC or GrlA component of topoisomerases. GyrA or GyrB components of gyrase</td>
<td>Mutations in QRDR region reducing affinity of enzyme-DNA complex for quinolones</td>
<td>C</td>
</tr>
<tr>
<td>E.g. ciprofloxacin</td>
<td><em>gyrA or gyrB</em></td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Aminoglycoside modifying enzymes (aac)</td>
<td>Acetyltransferase, Phosphotransferase</td>
<td>Modification of Aminoglycosides</td>
<td>Pl, Pl-Tn</td>
</tr>
<tr>
<td>E.g. kanamycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrofolate reductase inhibitors</td>
<td><em>dfrB</em></td>
<td>Dihydrofolate reductase (DHFR)</td>
<td>Reduced affinity for DHFR</td>
<td>C</td>
</tr>
<tr>
<td>E.g. Trimethoprim</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfonamides</td>
<td><em>sulA</em></td>
<td>Dihydropteroate synthase</td>
<td>Excess production of p-aminobenzoic acid by enzyme</td>
<td>C</td>
</tr>
<tr>
<td>E.g. sulfamethoxazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazolidinones</td>
<td><em>rrn</em></td>
<td>23SrRNA</td>
<td>Mutation in domain V of 23S rRNA component of 50S ribosome</td>
<td>C</td>
</tr>
<tr>
<td>E.g. linezolid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptogramins</td>
<td><em>ermA, ermB, ermC</em></td>
<td>Ribosomal methylases Acetyltransferases</td>
<td>Reduced binding of 23S Enzymatic modification of dalfopristin</td>
<td>Pl, C</td>
</tr>
<tr>
<td>E.g. Quinupristin-dalfopristin</td>
<td><em>vat, vatB</em></td>
<td></td>
<td></td>
<td>Pl</td>
</tr>
</tbody>
</table>

Table 4: Antibiotic resistance mechanisms seen in *S. aureus*. Examples of resistance to selected antibiotics. P=plasmid, C=chromosome, Tn=transposon, QRDR=quinolone resistance determining region. (Table adapted from Lowy, 2003; Werner et al, 2008).
There are currently several approaches to increasing antibacterial efficacy of drugs including combining one or more drugs to act in synergy, modification of existing antibiotics, and genomic approaches to find novel agents (Zinner, 2007; White and Kell, 2004). Drugs that affect multiple target sites such as multiple PBP may be preferred in the future and it is important that the target is selectively different functionally or structurally from human molecules. There are other criteria a new drug must fulfill such as effective distribution within the patient, ease of assay, low mutational potential and whether mode of action is bacteriostatic or bactericidal (Ohlsen and Lorenz, 2007). Bacteriostatic drugs are those that inhibit bacterial growth but do not kill the, bactericidal drugs kill the target bacteria. There has been some research into potentially novel antibacterial agents effective against *S. aureus*, including anti-staphylococcal plant products (Gibbons, 2004). There has also been research into novel target sites within *S. aureus* against which potential future antimicrobial agents could be sought (Table 5).

Some of the target sites identified have only been investigated *in vitro* and not *in vivo*, but none the less they are suitable for further investigation. From on-going work in UWIC (Henriques, 2006) the effect of manuka on *S. aureus* seems to be associated with failure to complete cell division. This study was aimed at investigating the target site and the mode of action of manuka honey on *S. aureus in vitro.*
<table>
<thead>
<tr>
<th>Target</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty Acid Synthesis</strong></td>
<td></td>
</tr>
<tr>
<td>Fabl</td>
<td>Enoyl-acyl carrier protein reductase</td>
</tr>
<tr>
<td>FabF/H</td>
<td>B-ketoacyl-(acyl carrier protein) synthase I/II</td>
</tr>
<tr>
<td><strong>DNA replication</strong></td>
<td></td>
</tr>
<tr>
<td>GyrA</td>
<td>DNA-gyrase</td>
</tr>
<tr>
<td>ParE</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td><strong>Protein modification</strong></td>
<td></td>
</tr>
<tr>
<td>Pdf</td>
<td>Peptide deformylase</td>
</tr>
<tr>
<td>tRNAsynthesis</td>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td><strong>Peptidoglycan synthesis</strong></td>
<td></td>
</tr>
<tr>
<td>PBP2</td>
<td>Peptidoglycan glycosyltransferases</td>
</tr>
<tr>
<td>MurB</td>
<td>UDP-N-acetylglucosamine-enolpyruvyl reductase</td>
</tr>
<tr>
<td>FmhB / FemAB</td>
<td>Pentaglycine interpeptide biosynthesis</td>
</tr>
<tr>
<td>Ddl</td>
<td>D-alanine:D-alanine ligase</td>
</tr>
<tr>
<td><strong>Regulation</strong></td>
<td></td>
</tr>
<tr>
<td>YycG/YycF</td>
<td>Autolysis</td>
</tr>
<tr>
<td>Gcp</td>
<td>Glycoprotease, autolysis</td>
</tr>
<tr>
<td><strong>Protein secretion</strong></td>
<td></td>
</tr>
<tr>
<td>SpsB</td>
<td>Signal peptidase</td>
</tr>
<tr>
<td><strong>Cell division</strong></td>
<td></td>
</tr>
<tr>
<td>FtsZ</td>
<td>GTPase</td>
</tr>
<tr>
<td><strong>Teichoic acid biosynthesis</strong></td>
<td></td>
</tr>
<tr>
<td>TarB, TarD, TarF, TarI, TarH</td>
<td>Teichoic acid polymer formation</td>
</tr>
<tr>
<td><strong>Stress response</strong></td>
<td></td>
</tr>
<tr>
<td>TrxA</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxB</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>ClpP</td>
<td>Proteolytic component of Clp complex</td>
</tr>
<tr>
<td>LigA</td>
<td>DNA ligase</td>
</tr>
</tbody>
</table>

Table 5: Potential novel targets for antibacterial agents in *S. aureus*. The target and function that could be targeted in a new therapy. Table adapted from Ohlsen and Lorenz (2007).
1.4 List of Objectives:

The overall objective for the study was to investigate the mode of action of manuka honey on the cell cycle of MRSA. The objectives for each of the respective stages of the study were as follows the chapter in which they were addressed are given in brackets:

To confirm the effect manuka honey on the viability of several strains of staphylococci and to determine which would be most suitable for use in further experiments (Chapter 2).

To observe the effect on the cellular structure of EMRSA-15 with a view to confirming cell division as a potential target site (Chapter 3).

To determine whether morphological changes induced by honey were time and dose dependant (Chapter 3)

To investigate changes in murein hydrolase function in MRSA in response to exposure to 10% (w/v) of manuka honey for up to 24 hours, using enzyme assays and zymography (Chapter 4).

To investigate changes in the cellular localization of FtsZ in MRSA in response to treatment with manuka honey (Chapter 5).

To investigate changes in cellular localization of Endo-B-N-Acetylglucosaminidase in MRSA in response to treatment with manuka honey (Chapter 6).

To observe the differences in protein expression between untreated, manuka honey treated, MGO treated and artificial honey treated EMRSA-15 four hours after treatment, using 2D gel electrophoresis and to determine whether any protein changes detected by 2-D electrophoresis might explain any of the structural changes seen in electron micrographs (Chapter 7).

To investigate the efficacy of a selection of honey impregnated dressings against a selection of wound infecting bacteria in vitro (Chapter 8).
2. An *in vitro* Study into the Antibacterial Efficacy of Honey Containing Dressings

2.1 Introduction

Honey has been since ancient times as not only a food source but also a medicinal product. It was used routinely in British hospitals up until the 1970s, however with the advent of antibiotics use of honey was reduced and eventually lost from mainstream medicine (Robson, 2005). Since the evolution of antibiotic resistance there has been a growing need for wound management products that can act as an alternative to antibiotics. Honey has several advantages as a wound therapy product; it has broad spectrum antimicrobial activity against bacteria, protozoa and some viruses (Al-Waili and Saloom, 2004; Cooper *et al*, 1999; Cooper *et al*, 2002; Willix *et al*, 1992; Simon *et al*, 2008). It has also been reported to improve wound healing, stimulate wound healing factors, removing ‘sloughy’ tissue and reduce wound odour (Gethin and Cowman, 2008; van Weyden, 2003). There are several reasons for the antimicrobial action, honey has a high sugar content leading to high osmolarity in the wound removing available water for bacteria, and it also has a low pH (Molan 1992).

Honey wound products have now been on the market in the UK since 2004 and several brands are available. Licensed CE wound care products are available on drug tariff by prescription, as well as tubes and sterile honey and ointments containing honey over the counter in pharmacies. Not all of the dressings produced are the same; they are made of differing materials: gauze, tulle, mesh, polyethylenevinylacetate or hydrogel, with different types of honey (for example *Leptospermum* honeys such as manuka and jellybush, whereas
others contain buckwheat, multi floral Chilean or unspecified honeys). Some of the
dressings also contain differing formulations adding components such as aloe vera,
vitamins and cod liver oil. Some honeys generate hydrogen peroxide on dilution and others
contain phytochemicals (Bogdanov et al, 2004; Molan 1992). There are many varying
honey devices now available on the market and these differing products need comparative
analysis to ensure that informed decisions can be made by healthcare professionals during
selection for clinical practice.

During this thesis manuka honey has been used as whole, undiluted honey. In practice such
may be applied topically to wounds directly from sterile tubes, but honey may also be
supplied in products it has been impregnated into medical devices such as dressings or
plasters. So although manuka honey has been investigated as a pure substance, it is
important to investigate how its formulation into medical devices affects antimicrobial
activity, and also how other honey impregnated devices compare to those impregnated with
manuka honey.

Wounds occur as a result of illness, accidents and operations in everyday life and can
become infected. Sometimes they develop into chronic wounds. Healthcare associated
infections are a major problem in the health care environment, especially with an increase
in the prevalence of antibiotic resistance (Bourn, 2004). These infections can lead to
delayed healing and increased morbidity, hospital costs and increased risk of spread of
infection.

There are many types of wound dressing available as different formulations aimed at
controlling or reducing wound infection. In recent years numerous honey based wound
care products have become readily available on the market. There is much evidence for honey as an antibacterial and wound healing agent and therefore being able to impregnate it into dressings is desirable (Molan and Betts, 2004; Maeda et al, 2008).

It is important however that these products are thoroughly tested to compare efficacy of the product against a wide range of wound infecting pathogens. Such studies will aid medical practitioners in making informed decisions on the correct product to use for each clinical case. These in vitro studies were designed to allow a relatively uncomplicated comparison of efficacy between antibacterial devices and to evaluate their effectiveness as barriers to bacterial spread.

2.2 Aim

To investigate the efficacy of a selection of honey impregnated dressing against a selection of wound infecting bacteria in vitro.

2.3 Methods

Strains used in these tests were Staphylococcus aureus NCTC 6571, Meticillin-resistant Staphylococcus aureus NCTC 13142 (EMRSA-15) and Pseudomonas aeruginosa (a clinical isolate from our collection at UWIC: LE08).

Seven different commercially available honey impregnated dressings (labelled 1-7) were used in a preliminary study where, zones of inhibition were tested. Dressings 3, 4 and 7 were used in a further study involving challenge with EMRSA -15. The dressings utilised were:
1. *Leptospermum* impregnated calcium alginate (Apinate, Comvita, UK)

2. *Leptospermum* impregnated calcium alginate (Algivon, Advancis Medical)

3. *Leptospermum* impregnated calcium alginate (Medihoney, Derma Science)

4. *Leptospermum* impregnated tulle (Activon Tulle, Advancis Medical)

5. American buckwheat honey impregnated mesh (Meldra, MedEllens)

6. Chilean multi-floral honey impregnated mesh (Honeysoft, Dermaprof)

7. Unspecified honey impregnated hydrogel (Mesitran, UnaMedical)

All were gifts from the companies directly or were collected at trade exhibitions at wound care conferences. All were within their stated expiry dates.

### 2.3.1 The zone of inhibition assay:

This test estimates the antimicrobial activity of components released from dressings. We adapted a standard technique used to determine the antimicrobial sensitivity (Thomas & McCubin, 2003).

Test cell suspensions were prepared from 24 hour nutrient broth (Oxoid) cultures of each organism, diluted with sterile broth to give optical density readings between 0.5 and 1 using a spectrophotometer (Cecil Instruments, Cambridge, UK) at wavelength 540 nm. 0.2 ml of the test suspension was inoculated into 200 ml of sterile, molten Tryptic Soy Agar (TSA) (Oxoid, Cambridge, UK) gently mixed, poured into large square assay plates (Corning, Sigma Aldritch, UK) and allowed to set. Samples of each of the dressings were cut into 2 x 2 cm squares and placed evenly onto the surface of the dried plates, left at room temperature for one hour then incubated over night at 37°C. After incubation plates were examined for zones of inhibition, the length and width were measured (mm) using digital
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callipers and the average was calculated from these readings. Each experiment was done in
duplicate on three occasions. The extent of each zone of inhibition was measured twice (at
right angles) across the middle of each dressing and the mean calculated.

2.3.2 The barrier effect of dressings:
The dressing was then removed from the surface of the agar plate using sterile forceps and
placed onto the surface of a sterile TSA plate and left for 5 minutes in the same orientation.
A second set of sterile forceps was used to lift the dressing and invert it onto the surface of
another sterile TSA plate. After five minutes the dressing was discarded and these plates
were incubated at 37°C for 24 hours (Edwards-Jones, 2006). Aseptic technique was used
throughout; experiments were performed in duplicate on three separate occasions. Results
were recorded after the 24 hour incubation and recorded as no growth (NG), light growth
(+), growth (++) or heavy growth (+++).

2.3.3 Challenge Test
An EMRSA-15 starter culture (10 ml) was grown at 37°C overnight and a loopful
inoculated into 25 ml nutrient broth (Oxoid, Cambridge, UK) and incubated overnight at
37°C in a shaking water bath at 120 rpm. Cells were harvested by centrifugation at 3000 g
for 10 minutes (MSE Harrier 15/80 centrifuge, Sanyo) at room temperature and re-
suspended in sterile, quarter strength Ringers to an optical density of 0.5 at 550 nm with a
Cecil spectrophotometer. Cell suspensions were tested within 10 minutes of preparing the
diluted inoculum and numbers were estimated by a total viable count which was performed
immediately after preparation and before the start of each experiment.
Into a sterile Petri dish 3 layers of sterile gauze approximately 4 x 4 cm were placed, onto this a 2 x 2 cm sample of one of the dressings being tested (Activon Tulle, Medihoney or Mesitran ) was placed. In total, 9 of these Petri dishes were prepared for each of the three types of dressing.

All dressings were inoculated at time 0 with 200 µl EMRSA-15 which was spread evenly over the dressing using a sterile plastic spreader. The number of viable cells at each time point was determined by aseptically removing a dressing from its Petri dish into 9 ml sterile maximum recovery diluent (Oxoid, Cambridge, UK). This was vortexed at maximum speed for 15 seconds on a mechanical mixer before total viable counts were performed.

Serial decimal dilutions were prepared in the sterile maximum recovery diluent and then plated onto nutrient agar (Oxoid ) following the Miles and Misra method. These plates were incubated at 37°C for 48 hours, before colonies were counted and total viable counts for surviving bacteria on each dressing were calculated. The experiment was performed on three separate occasions.

2.4 Results

2.4.1 Zone of Inhibition test

The buckwheat and multi-floral honey impregnated dressings showed no zones of inhibition against any species of test bacteria, although the *Leptospermum* honey impregnated dressings gave zones of inhibition against all three species of bacteria (Figure
6). The staphylococci were more sensitive to the honey than the *Pseudomonas aeruginosa* (Figure 7).

**Figure 6. Zones of inhibition caused by six wound dressings against EMRSA-15:** Zones of inhibitions observed around dressings 1-4 the *Leptospermum* honey impregnated dressings. No zones of inhibition seen around dressings 5 and 6, the buckwheat honey and multi-floral Chilean honey impregnated dressings.
**Figure 7: Zones of inhibition caused by each of six dressings against three species of bacteria.** White filled bars indicated *Staphylococcus aureus* most sensitive to the dressings giving largest zones of inhibition. Grey bars indicates zones of inhibition against EMRSA-15. Black filled bars indicate zones of inhibition seen against *Pseudomonas aeruginosa*, much smaller zones seen, indicating less sensitivity to the honey impregnated dressings, No zones seen from non manuka dressings.
2.4.2 Barrier Effect

The previous experiment shows the antibacterial effect of the honey formulated within the dressing. However it does not show whether bacteria are able to migrate into the dressing or if dressing is acting as a barrier and preventing migration right through to the top side of the dressing and potentially generating a risk of cross-infection.

As there was no observed growth of either of the strains of staphylococci on the upper surface of the calcium alginate dressings, indicating that all three dressings were acting as a barrier preventing migration of bacteria from the surface of the TSA plate to the upper surface of the dressing (Table 6). The buckwheat honey and multi-floral honey impregnated dressings allowed translocation of bacteria from the surface of the plate to the upper surface of the dressings, indicating that these dressing were not as effective as a barrier (Table 6). The *Pseudomonas aeruginosa* penetrated through to the upper surface of all the dressings (Table 6). These observations suggest that the mode of action of most of the dressings was not bactericidal. Dressings which had caused the formation of large zones of inhibition on bioassay plates still retained viable organisms on their undersides which grew when once transferred onto fresh agar plates (Figure 8). Hence the mode of inhibition was deduced to be bacteriostatic. This was clearly seen as both the top and the underside of every dressing was placed onto a fresh agar plate to be tested. The exception to this was *Leptospermum* honey impregnated calcium alginate number two which did not give rise to any colonies once transferred onto fresh plates, therefore demonstrating a bactericidal mode of action (Figure 9) (Table 7). This mode of action was only seen against *S. aureus*, not seen with either EMRSA or the *Pseudomonas*. The figures presented are displayed to illustrate the various modes of action seen after treatment with the different types of honey impregnated dressings.
Table 6. Semi-quantitative growth of 3 species of bacteria on the upper surface of six dressings (barrier effect): NG = no growth from upper surface of dressing after subculture onto new medium; + = light growth, ++ = growth, +++ = heavy growth. All three Leptospermum honey impregnated calcium alginate were effective barriers against strikethrough of both Staphylococci strains. NG = less than one bacteria per ml.

<table>
<thead>
<tr>
<th>Dressings</th>
<th>Oxford Staphylococcus aureus</th>
<th>EMRSA-15</th>
<th>Ps. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptospermum impregnated</td>
<td>NG</td>
<td>NG</td>
<td>+++</td>
</tr>
<tr>
<td>calcium alginate 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptospermum impregnated</td>
<td>NG</td>
<td>NG</td>
<td>+++</td>
</tr>
<tr>
<td>calcium alginate 2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Leptospermum impregnated</td>
<td>NG</td>
<td>NG</td>
<td>+++</td>
</tr>
<tr>
<td>calcium alginate 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptospermum impregnated tulle</td>
<td>NG</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Buckwheat impregnated mesh</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multi-floral impregnated</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>mesh 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. The antimicrobial effect of dressing 4, Leptospermum impregnated Tulle against *Staphylococcus aureus*: At 24 hours *Leptospermum* honey impregnated tulle shows zone of inhibition against *S. aureus* (picture on left). After underside of dressing (top plate) and upper surface of dressing (bottom plate) were observed effect was shown to be bacteriostatic.
Figure 9. The antimicrobial effect of dressing 2, *Leptospermum* honey impregnated calcium alginate 2 against *Staphylococcus aureus*: At 24 hours *Leptospermum* impregnated calcium alginate 2 shows zone of inhibition against *S. aureus* (picture on left) After sub culture of dressing underside (top plate) and upper surface of dressing (bottom plate) effect was shown to be bactericidal.
Table 7. Semi-quantitative growth of three organisms on the lower surface of six dressings:

<table>
<thead>
<tr>
<th>Dressings</th>
<th>Oxford <em>Staphylococcus aureus</em></th>
<th>EMRSA-15</th>
<th>Ps. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptospermum</em> impregnated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>calcium alginate 1</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Leptospermum</em> impregnated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>calcium alginate 2</td>
<td>NG</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><em>Leptospermum</em> impregnated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>calcium alginate 3</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><em>Leptospermum</em> impregnated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tulle</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Buckwheat impregnated mesh 1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Multi-floral impregnated mesh 2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 7. Semi-quantitative growth of three organisms on the lower surface of six dressings: NG = no growth under dressing after subculture onto new medium; + = light growth, ++ = growth, +++ = heavy growth. Mode of action shown to be bacteriostatic from all dressings against all species of bacteria except *Leptospermum* honey impregnated calcium alginate 2 showing bactericidal action against *S. aureus*. 
2.4.3 Challenge Test

This test estimates the ability of the dressing to reduce bacterial viability and showed that the three types of dressing tested had varying efficacies against EMRSA-15. Activon Tulle (Advancis Medical, Nottingham, UK) appeared to be the most effective dressing against EMRSA-15, followed by Medihoney (Slough, UK) dressing. The Mesitran (Worcestershire, UK) having a much reduced effect compared to the first two dressings (Figure 10). The decimal log reductions after 240 minutes were 4.5, 4.2 and 1.6 for Activon Tulle, Medihoney and Mesitran respectively. The Activon Tulle and Medihoney reduced total number of culturable cells to undetectable levels after 360 minutes, but viable cells remained with Mesitran dressing at this time. However, after 24 hours test organisms were at undetectable levels with Mesitran, too. This test shows that the inhibition rates from each type of dressing differed in the conditions of the test.
Antimicrobial activity of honey dressings against EMRSA-15

Figure 10. Challenge test, Antibacterial efficacy of three honey impregnated dressings against EMRSA-15: The efficacy of honey impregnated dressings in inhibiting EMRSA-15.
2.5 Discussion

The results of this brief study demonstrate that all of the *Leptospermum* honey impregnated honey dressings possess sufficient antimicrobial activity to inhibit the growth of all the organisms tested. The broad spectrum activity of *Leptospermum* honey is well documented but the lack of zones of inhibition from the buckwheat honey and multifloral honey impregnated dressings reinforces the importance of correct dressing selection for each wound individually. Not all honey impregnated dressings make antibacterial claims and the difference to efficacy that the formulation of honey in dressings makes is clearly seen from the varying zone sizes of the four dressings impregnated with *Leptospermum* honey. The smaller zones seen with *P. aeruginosa* could reflect the rapid growth of the organism during the time taken for the honey to be released into the agar surface (Thorn *et al.*, 2005) as well as a higher MIC. The importance of careful dressing selection is again emphasized by the results of the barrier test; the *Leptospermum* honey impregnated tulle gave large zones of inhibition similar to those of the alginates, however only the alginates prevented migration of the bacteria through the dressing therefore making honey impregnated alginates a more appropriate dressing if trying to prevent the spread of antibiotic resistant bacteria into the environment or between people (Edwards-Jones, 2006).

The methods described in the assays using zone of inhibition and the barrier tests provided semi quantifiable data (no kill rates) and showed that the honey impregnated dressings had differing modes of action for the bacteria tested (Holland and Davis, 1985). Both bactericidal and bacteriostatic modes of action were observed. The preliminary study using challenge tests has reinforced the differences in efficacy between honey impregnated
dressings. The differences seen in these dressings could be due to several factors. The concentration and type of honey within each type of dressing differs, as does the material used for impregnation, the formulation of honey and possible inconsistent dispersal of the honey within the dressing could be a factor. In the case of the three dressings not exhibiting barrier effect against *Pseudomonas aeruginosa* species there is an additional possibility, that the motile bacteria could have penetrated the dressing from the agar plate by swimming inwards from the edge, rather than directly through the dressing.

There were several limitations to these studies. The bacteria tested here were grown in pure culture which did not closely represent the polymicrobial environment of a wound, the pH and oxygen availability of the wound would also have been different to that of the agar plates (Cooper, 2007). Care must be taken when taking *in vitro* data into the clinical environment as this study investigated the antimicrobial properties of these dressings therefore not taking into account the wound healing properties or fluid handling capacity of the dressings. The results here show that *Leptospermum* impregnated honey dressings have more effective antimicrobial properties than the buckwheat honey impregnated ones and that the alginates were more effective as barriers, these results could vary if tested with a different model *in vitro* or if tested *in vivo*, as there would be more variables (Cooper, 2008).

There is a need for more *in vitro* tests to be carried out as well as randomised controlled clinical trials before efficacy can be truly compared.
3 Inhibition of MRSA by manuka honey

3.1 Introduction

For antimicrobial agents to be used safely and efficiently it is important for the mode of action of the agent to be understood. Honey has been used for hundreds of years as a treatment for ailments but has only recently been accepted into use in modern medicine and there is no one clear mechanism of action known for honey.

The first manuka honey impregnated dressing was introduced into the British market in 2004 and since then honey impregnated plasters and other devices have become available via prescription. Despite this it is important that there is further research into the action of manuka honey on relevant wound infecting pathogens (Molan & Betts, 2004).

For this study into the mode of action of manuka honey against *Staphylococcus aureus*, several strains were used in an initial investigation. Staphylococci are representative of common wound infecting bacteria; since the advent of meticillin resistance strains of MRSA have become some of the hardest bacterial species to control and eradicate, especially in the hospital environment.

*Staphylococcus aureus* is carried by 30% of the population, being ubiquitously associated with human skin and it can become an opportunistic infective agent when a break in the skin occurs (Lowy, 2003). There has been a worrying increase in the incidence of meticillin-resistant *S. aureus* in both the community and hospital over the last decade due to continued selection by the use of antibiotics. These bacteria have proved very difficult to
treat as they carry resistance to more than one type of antibiotic agent, the latest one being vancomycin. Hence vancomycin-resistant *S. aureus* or VRSA now exists (Ang, Ezike & Asmar, 2004). Due to the increase in multi-resistant bacteria it is becoming increasingly important to test for new antimicrobial compounds, against laboratory and clinical strains as clinical isolates have been subjected to environmental stresses that may have altered their resistance profiles.

For manuka honey to be accepted as a main stream form of treatment, it is important that its mechanism and kinetics of its inhibition be elucidated. This information can be collected in the form of time to kill curves, minimum inhibitory concentration, and minimum bactericidal concentration (Stratton, 2003). Although some data on staphylococci has been published (Cooper and Molan, 1999; Cooper *et al.*, 2002; French *et al.*, 2005), time kill curves are not yet available.

When antimicrobial testing was first performed there was no standardisation between laboratories and results could vary greatly due to different concentrations of inoculum used, different media and the different antimicrobial agents used (Piddock, 1990). Accurate antimicrobial testing is essential to allow physicians to devise suitable treatment strategies, so once the problem was recognised in 1952, standard techniques were established. Revised methods for antimicrobial testing are published every 3 years by the Clinical Laboratory Standards Institute, CLSI (formerly known as NCCLS) in the USA; methods are also published by the British Society for Antimicrobials and Chemotherapy (BSAC). These methods thoroughly describe inoculum size, media and antimicrobial dilutions that should be used, as well as providing reference tables and control protocols to assess resistance or susceptibility to a particular antimicrobial agent (Piddock, 1990).
It is probable that resistance or susceptibility observed in vitro is likely to be present in vivo, although it is not as clear with susceptibility as there are many substances that can cause interference with antimicrobial agents in vivo that are not present in standard laboratory tests (Varaldo, 2002).

One of the most important questions that can be asked of an antimicrobial agent is whether its effect on its target organism is to cause it to lose viability or not. (Laflamme et al, 2004). For honey to be accepted and routinely used as an antimicrobial of clinical significance it is important to provide data that show the effect of honey on the growth of bacteria, whether it is bactericidal or bacteriostatic. This can be tested using time to kill, MIC/MBC (Minimum inhibitory concentration and minimum bactericidal concentration) and confocal microscopy. The CLSI and BSAC methods for susceptibility testing against antimicrobial agents are standard tests used in hospitals and laboratories all over the world. The minimum inhibitory concentration can be determined in both solid and liquid media and is the lowest concentration of the antimicrobial agent (honey) that prevents growth of the organism.

The CLSI method for determining MIC has been described by Wikler et al 2006, the BSAC method is similar but utilises Iso-Sensitest Broth (Oxoid) (www.bsac.org.uk).

The incidence of Staphylococcus aureus related bacteraemia due to MRSA rose from 1-2% to around 40% between 1990 and 1992, this rise correlates with the increase in emergence of EMRSA-15 and EMRSA-16 (Duckworth et al, 1998; Johnson et al, 2001). Britain is now in the top five European countries with high levels of MRSA incidence and it has been
suggested that EMRSA-15 and EMRSA-16 maybe more pathogenic than other clones (Gould et al, 2008). Therefore any new antimicrobial that could successfully kill these strains would be of medical relevance for topical wound infections.

3.2 Aim

The aim of the experiments presented in this chapter was to observe what effect manuka honey had on viability of several strains of staphylococci and to determine which test organism would be most suitable for use in further experiments.

3.3 Methods

3.3.1 Strains

Five type cultures were used: *Staphylococcus aureus* ATCC 10017, *Staphylococcus aureus* NCTC 6571, a clinical isolate that was isolated by Professor Rose Cooper from a patient successfully treated with manuka honey in a local out-patient clinic (Natarajan et al. 2001), labelled MRSA-18 within a collection stored at -80ºC at University of Wales Institute, Cardiff (UWIC), Epidemic meticillin-resistant *Staphylococcus aureus* 15 NCTC 13142 (referred to as EMRSA-15 throughout rest of thesis) and Epidemic meticillin-resistant *Staphylococcus aureus* -16 NCTC 13143.

3.3.2 Honey

The honey used was a sample of non sterile non peroxide producing manuka honey (M109), which has a phenol equivalent of 18 donated by Professor P. Molan from the University of Waikato, New Zealand. The honey samples were filtered through a disposable 0.22 µm disposable syringe filter (Millex-GV, Millipore) to ensure sterility of
honey. Overnight cultures were obtained by inoculating 1 colony of the appropriate bacteria into 10 ml of the broth being used for the experiment; this was then cultured overnight at 37°C.

Artificial honey was prepared by adding 1.5 g sucrose to 17 ml deionised water, mixing and adding 7.5 g maltose, 40.5 g fructose and 33.5 g D+glucose (Oxoid). This was thoroughly mixed and used throughout the study.

### 3.3.3 Growth Curves

Growth Curves were performed by inoculating 20 ml of Tryptic Soy Broth (TSB) (Oxoid, Cambridge, UK) with 100 µl of an overnight culture and incubating at 37 ºC in a shaking water bath (120 rpm). Time of inoculation was noted (time 0) and 2 ml samples of culture were removed at known time intervals and absorbance at 520 nm determined in a Cecil spectrophotometer. Samples were collected at multiple time points so that exponential and stationary growth phases could be identified when absorbance was plotted against time. Growth curves were only performed on EMRSA-15 as it had been decided that this was the bacterium to used in future experiments.

### 3.3.4 Minimum Inhibitory Concentration (MIC) in Microtiter plates

Briefly the wells in a flat bottomed 96 well Microtiter plate were inoculated with a final volume of 200 µl, which was made up of 100 µl double strength nutrient broth plus 100 µl of double the desired final concentration of honey made up in the appropriate diluent, which was sterile deionised water for total activity. To each of the wells except the no
growth control well 1 µl of overnight culture was added, inocula used were roughly $10^6$ cfu/per ml. The plate was then incubated overnight at 37°C before turbidity was measured at 520 nm in a plate reader (MRX Revelation, Dynex). Total viable cell counts (TVCs) were conducted to retrospectively determine population size; the Miles Misra surface drop count method was used (Drabu & Blakemore 1990; Levett 1991; Tweats, Green & Muriel 1981). Both positive and negative controls (broth and inoculum and honey and no inoculum) were used. Those wells with the lowest concentration of honey showing no growth after 24 hours were considered to be the MIC.

### 3.3.5 Minimum Bactericidal Concentration (MBC)

A 20 µl volume of cells were taken from those wells in the above plates showing no growth and plated onto nutrient agar plates (Oxoid). These plates were incubated overnight at 37°C, the plates with the lowest concentration of honey showing no growth after this time was recorded as the MBC.

### 3.3.6 Time Kill Curves

From an overnight culture of EMRSA-15 (selected after the results of the MIC/MBC tests in nutrient broth) (Oxoid) a 40 µl sample was inoculated into 20 ml nutrient broth alone, or nutrient broth containing 2.5%, 5%, 10%, or 20 % (w/v) manuka honey, this was done by weighing out the appropriate amount of manuka honey into the final volume of broth and then dissolving it by agitation. Samples were removed at known time intervals and total culturable counts were obtained using Miles and Misra surface drop method (Drabu & Blakemore 1990; Levett 1991; Tweats, Green & Muriel 1981), using ¼ strength ringers as a diluent. Time-kill curves were plotted.
3.3.7 Confocal Microscopy

This experiment was conducted by adding one colony of EMRSA-15 to each of three universal containers holding 10 ml TSB (Oxoid), incubated overnight at 37°C. Overnight cultures were then added to 270 ml TSB and incubated at 37°C in orbital incubator for 24 hours. Cells were then harvested by centrifugation at 8000 g (Sorvall RC5B, DuPont Instruments) for 15 minutes and resuspended in 3 ml TSB. One millilitre of the resuspended cells was then added to each of three flasks containing 99 ml TSB, 99 ml TSB containing 10% (w/v) manuka honey or 99 ml TSB containing 10% (w/v) artificial honey.

After inoculation (time 0) and every 30 minutes for a set time period a 1 ml sample was taken from each flask and spun for 2 minutes at 13000 rpm (MSE Microcentaur, Sanyo), cells were then resuspended in 1 ml 0.85% (w/v) sterile NaCl. A Baclight live/dead bacterial viability kit (Invitrogen, Paisley, UK) was then used and 1.5 µl of component A and 1.5 µl component B were thoroughly mixed together before being added to a 1 ml sample of cells. The cells were then incubated in the dark for 15 minutes at room temperature before 5 µl samples were trapped between a slide and a 22 mm x 22 mm coverslip, these were immediately observed using epifluorescence optics, on a Leica TCS SP2 AOBS spectral confocal microscope, operated by Tony Hayes at Cardiff University, this experiment was conducted over several weeks.
3.4 Results

3.4.1 Growth Curves

The growth curve showed that the exponential phase for EMRSA-15 in TSB occurred between 60 and 375 minutes. This experiment was performed in triplicate and the mean of these results is represented in Figure 11. The generation time (or cell cycle) of EMRSA-15 in nutrient broth at 37°C was 30 minutes; similarly generation times in nutrient broth containing 10% (w/v) artificial honey solution and 2.5% (w/v) manuka honey were 30 minutes, figures not presented here.

3.4.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in Microtiter plates

The results of the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) determination of manuka honey M109 are shown in Table 8.
Figure 11: Growth curve of EMRSA – 15 in TSB: The exponential phase was observed between 60 - 375 minutes. The generation time (or cell cycle time) of EMRSA-15 in nutrient broth at 37°C was 30 mins; similar generation times were seen in nutrient broth containing 10% (w/v) artificial honey solution and 2.5% w/v manuka honey were 30 mins.
3.4.3 Time Kill Curves

For the time to kill curves MRSA was tested in NB containing 2.5%, 5%, 10%, 20% w/v manuka honey. From the data presented in the figure below, the highest concentration of manuka honey tested was more than three times the MBC. The flasks containing 10% artificial honey in NB and nutrient broth alone were used as controls (Figure 12).

Table 8: Showing MIC/MBC for 5 strains of bacteria when tested in nutrient broth with manuka honey (M109). SD= standard deviation, n = 8, the number of times the test was carried out. There was no significant difference between the MIC or MBCs of any of the strains.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>MIC (%w/v)</th>
<th>SD</th>
<th>MBC (%w/v)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus ACTCCC 10017</td>
<td>2.5</td>
<td>0.003</td>
<td>4.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Staphylococcus aureus NCTC 6571</td>
<td>2.5</td>
<td>0.01</td>
<td>4.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Epidemic methicillin-resistant <em>Staphylococcus aureus</em> 15</td>
<td>2.5</td>
<td>0.02</td>
<td>5</td>
<td>0.002</td>
</tr>
<tr>
<td>Epidemic methicillin-resistant <em>Staphylococcus aureus</em> 16</td>
<td>2.5</td>
<td>0.002</td>
<td>5</td>
<td>0.001</td>
</tr>
<tr>
<td>MRSA - 18</td>
<td>2.5 – 3</td>
<td>0.002</td>
<td>5</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Figure 12: Time Kill Curve of EMRSA-15 treated with and without manuka honey: Time-kill curves of MRSA with and without honey (M109). Viable cell counts of EMRSA-15 NCTC 13142 incubated in nutrient broth (NB) (filled diamonds), NB containing 10% (w/v) artificial honey (filled triangles), NB containing 2.5% (w/v) manuka honey (filled squares), NB containing 5% (w/v) manuka honey (open squares), NB containing 10% (w/v) manuka honey (stars) and NB containing 20% (w/v) manuka honey (open circles).
3.4.4 Confocal Microscopy

There was no change over time in the percentage of dead cells observed using confocal microscopy in any of the samples examined. However there was a noticeable decrease in the numbers of cells seen in the honey treated samples throughout the time course compared to control cultures. There was also a difference in the appearance of those cells treated with manuka honey over time compared to NB alone and NB with 10% (w/v) artificial honey. Examples of confocal pictures captured at time 0 and time 180 are presented in Figures 13 - 18.
Figure 13: Confocal microscopy image showing control cells at T0: Live cells on left hand side of frame in green, dead cells on right hand side of frame in red.

Figure 14: Confocal microscopy image showing cells treated with artificial honey at T0: Live cells on left hand side of frame in green, dead cells on right hand side of frame in red.
Figure 15: Confocal microscopy image showing cells treated with manuka honey at T0: Live cells on left hand side of frame in green, dead cells on right hand side of frame in red.

Figure 16: Confocal microscopy image showing control cells at T180: Live cells on left hand side of frame in green, dead cells on right hand side of frame in red.
Figure 17: Confocal microscopy image showing cells treated with artificial honey at T180: Live cells on left hand side of frame in green, dead cells on right hand side of frame in red.

Figure 18: Confocal microscopy image showing cells treated with manuka honey at T180: Live cells on left hand side of frame in green, dead cells on right hand side of frame in red.
3.5 Discussion

Honey is now being considered as a possible alternative for antibiotics the treatment and prevention of localised wound infections. The antimicrobial activity of honey has been compared to phenol to give an idea of its potency; this is despite the fact that phenol is not generally used to treat wound infection, and is used instead as a disinfectant (Allen et al., 1991). The honeys being used in these experiments and those in other chapters were tested against phenol and this showed that the honeys used in all of the experiments had a phenol equivalent of 18% (w/v).

In later chapters M109 manuka honey was substituted with Activon manuka honey (Advancis Medical Ltd). The reason for swapping from M109 to Activon was due to Activon being available in a sterile form (it was a wound care product and had therefore been gamma irradiated to sterilise it) removing the need for filter sterilising the honey, which is a messy and time consuming step.

The initial work carried out here was done using nutrient broth but tryptic soy broth was used in later work. Theses two broths are non defined media and there is good reason for using them. Manuka honey is viewed as an antimicrobial agent potentially as an alternative to antibiotics in certain cases, therefore the environment in which it would be used, on the body, is an undefined one. The precise content of the treatment site would not be known and would contain variable amounts of proteins, carbohydrates and enzyme. Using defined media would not be representative of a wound environment, undefined media replicates this to a point as there may be things in this which interfere with the antimicrobial action of the honey much as components of wound fluid may increase or decrease the antimicrobial
activity of honey. This situation is also complicated by the fact that the honey being used in these experiments is not a defined antimicrobial agent such as meticillin but contains many elements not all of which are known. There is potential for future work to be carried out supplementing the media with components found in the wound environment such as blood or plasma. Defined media such as isosensitest broth are advised for antibiotic susceptibility tested methods as components within undefined media such as calcium can interfere with the activity of the antibiotics decreasing or increasing activity.

The gold standard for determining the susceptibility of an organism to an antimicrobial substance has been to elucidate the minimum inhibitory concentration. There are traditionally several methods to choose from to do this, these include challenging the bacteria on agar or in broth in tubes or in microtitre plates (Andrews, 2001; Wheat, 2001). In this case microtitre plates containing broth cultures were used, this was because a more accurate result is obtained from the plate reader than from results taken by eye in the tube dilution method, also it allowed an increased number of replicates and minimised the amount of materials being used.

It is preferable, where possible, to use antimicrobial therapy that has a bactericidal effect on the organism that it is targeting. This allows for complete removal of the challenge from the host and reduces the likelihood of recurrent infections from surviving or small colony variant bacteria once treatment is stopped and also lowers the risk of resistant horizontal gene transfer occurring (Laham et al, 2007; Dzidic and Bedekovic, 2003). The MBC/MIC ratio of given antimicrobials have been used as an indicator of the mode of action of the antimicrobial agent against the microbe it is tested on (Agnese et al, 2001; Barry and Fuchs, 1991; Saravolatz et al, 2007). If the MBC/MIC ratio is less than four the action of
the antimicrobial is said to be bactericidal, whereas anything with a ratio above four is regarded as being bacteriostatic. The results from the experiments carried out in this section show that manuka honey used in these experiments has a bactericidal effect on all of the strains it was tested on as the MBC/MIC was below 4 in all cases.

The time kill data (p 34) allowed the selection of a honey concentration to be used in future work. It indicated that as shown previously the minimum concentration of manuka honey to affect *S. aureus* or MRSA would have to be higher than the 2.5% (w/v) control used, those cells treated with 2.5% (w/v) showed no loss of viability. It also indicated that increasing the honey concentration above the minimum inhibitory concentration did not have an obvious increase in effect, the 5%, 10% and 20% populations all behaving in a similar manner, showing a similar loss in viability. Classical dose response curves are often used in antibiotic testing to determine the median lethal dose (LD$_{50}$) this is the dose of a substance required to kill half the population. There is no dose response curve for honey as there is more than one active component present, therefore making the relationship between dose and response non linear and skewing curve and therefore the results.

The laser scanning confocal microscopy was used in conjunction with a Live Dead Bac light kit. This is a relatively new technique used for evaluating the bacterial viability thought to give a more accurate estimate of viable bacteria in a population than the traditional plate count. The traditional plate count method is accepted to underestimate viable bacteria within a population as it only expresses those bacteria that will complete active division in the given culture conditions and does not include those which are unculturable (Laflamme *et al*, 2004). The assessment of viability of bacteria is based on membrane integrity, bacteria with intact membranes are viable and will be stained green by
SYT09 (a total nucleic acid stain), those with damaged membranes are stained red with propidium iodide and are deemed non viable. These dyes have been used in many different studies to look at viability of both staphylococcal and other species of bacterial cells (Berney et al, 2007; Filoche et al, 2007; O’Neill et al, 2004). What the confocal images seemed to show in this case was that the treatment of cells with 10% (w/v) manuka honey did lead to a reduction of cell numbers however there was no increase in the proportion of dead (red) cells seen. This suggests that cells might be dying in some way that does not initially involve damage to the cell membrane and the accumulation of non viable (dead) cells but that leads to total cell destruction/disintegration. This would explain the lack of red stained cells seen. This is supported by the fact that although it is assumed that bacteria with compromised membranes are dead or have reduced viability the reverse of this (cells with intact membranes are alive) is not necessarily true, it has been reported that intermediate states occur (Berney et al, 2007). Some bacteria with intact membranes once plated onto medium may not be able to reproduce and conversely those with damaged membranes counted as dead using this technique may recover and reproduce if plated on to media. The viability of cells would be best defined by using several different measures of viability such as enzyme activity, coupled with membrane permeability to reduce the limitations inherent within each individual method.

For MRSA cells exposed to inhibitory honey concentrations (manuka at 5, 10 and 20% w/v) the differences seen between the time kill curves (decrease in culturable cells) and the confocal microscopy (very few dead cells seen) was unexpected but could be due to the presence of viable but non culturable bacteria (VBNC). The VBNC state is one where the bacteria are unable to reproduce and grow on the media on which they would normally
produce colonies, however they are alive and are believed to be capable of renewed metabolic activity (Oliver, 2005).

This state was first reported in *Escherichia coli* and *Vibrio cholerae* reporting cells that were still alive but unable to be cultured (Xu *et al.*, 1982). This state is thought to be an adaptation of cells under environmental stress that would kill them if they did not undergo this change in state, such as; temperature, pH changes, osmotic concentration, and these stresses cause the physiological change that produces viable but non culturable colonies. Since this state was first reported in 1982 up to 60 bacteria are known to be able to enter this state, including human pathogens such as *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Shigella dysenteriae* (Oliver, 2005). It could be that inhibition of *Staphylococcus aureus* and MRSA by manuka honey altered the environmental conditions in a way which induced this phenotype of cell.
4. An Investigation into the Effects of Manuka Honey on the Structure of Meticillin resistant *Staphylococcus aureus*

**4.1 Introduction**

Inhibition of wound pathogens by honey has been demonstrated *in vitro*, with both antibiotic sensitive and resistant strains proving to be susceptible at relatively low concentrations (Cooper *et al*, 1999; Cooper *et al*, 2002; George and Cutting, 2007; Willix *et al*, 1992). There have also been several reports of the eradication of MRSA from wounds following topical application of honey (Natarajan *et al*, 2001; Eddy and Gideonson, 2005; Chambers, 2006; Blaser *et al*, 2007; Visavadia *et al*, 2008). Manuka honey has been shown to inhibit meticillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* with an MIC of less than 3% v/v (Cooper *et al*, 2002) and mode of inhibition is bactericidal (Henriques, 2006 and chapter 3).

As yet the mechanisms of action and target sites of manuka honey in MRSA are unclear. Preliminary experiments in our laboratory indicate that exposure of *Staphylococcus aureus* to 10 % (w/v) manuka honey for 4 hours caused an interruption to the cell cycle, at the stage of cytokinesis, increased numbers of cells with sepal components were seen (Henriques, 2006).

The benefit of using electron microscopy to study cytological effects of antimicrobial agents on *Staphylococcus aureus* to look for possible intracellular targets has been shown in numerous studies (Carson *et al*, 2002; Castillo *et al*, 2006; Lorian and Fernandes, 1999).
The use of electron microscopy allows the visualisation of any damage or abnormalities within the structure of the cell, such as blebs, lesions, leakage of cytoplasmic content, and growth of multiple cell walls (Brunskill et al., 1997). Also the presence of debris or intracellular components in the thin sections may indicate that the target site of an antimicrobial agent could be structurally related and causing cell wall damage. It could also indicate that the antimicrobial agent has caused activation of autolytic enzymes within the cell leading to cell lysis and death.

The work done by Henriques (2006) was limited in several ways. Cultures were grown and harvested cells were tested in buffer with and without manuka honey; it is possible that the absence of nutrients may have affected the viability of the cells and could have contributed to the effects seen. Also only one contact time (4 hours) was observed, so it was not known how quickly the observed effects took place and whether the effects would have changed over time. The use of only one honey concentration meant it was unknown whether the effect was dose dependant or if any changes would occur more strongly with a higher concentration of honey.

4.2 Aims

The aims were to observe the effect of differing concentrations of manuka on the cell structure of EMRSA-15 and to observe whether the effects were dose dependant or affected by length of exposure to the honey.
4.3 Methods

This part of the study occupied 7 months. The culture used was Epidemic meticillin-resistant *Staphylococcus aureus* 15 NCTC 13142 and the sample of manuka honey used was M109 that was kindly provided by Professor Molan of the University of Waikato in New Zealand. Essentially the experiment was initiated on Llandaff Campus at UWIC with cells exposed to varying concentrations of honey for known periods and fixed specimens transported to the Electron Microscope Unit at Cardiff University for processing for electron microscopy with the help of Dr A. Hann.

4.3.1 Collection of Cells for Electron Microscopy

Aseptic technique was used throughout. A single colony taken from a 24 hr plate culture of test organism was incubated overnight in 20 ml of sterile nutrient broth (Oxoid, Cambridge, UK) in a shaking (120 cycles/min) water bath at 37°C. The total volume of cells were spun down at 3000 g for 30 minutes, resuspended in nutrient broth and 6 equal volumes of inoculum were resuspended in each of six conical flasks containing 50 ml of nutrient both with either 2.5%, 5%, 10%, 20% w/v manuka honey, 10% w/v artificial honey or with no additions. For transmission electron microscopy (TEM), 4.5 ml samples were removed at 0, 30, 60, 90, 120, 240 and 1440 minutes, cells were spun down at 10000 g for 2 minutes and washed 3 times in 1.5 ml sodium phosphate buffer 0.1M, pH 7.4. After washing the cells were resuspended in 500 µl of a 3% glutaraldehyde solution (Fluka) for one hour at 4°C, and then washed twice in 1.5 ml sodium phosphate buffer before resuspension in 1 ml of the same phosphate buffer. At this point samples were transferred to Cardiff University for electron microscopy.
4.3.2 Preparation of Cells for Electron Microscopy

Cells were examined by transmission electron microscopy following the method described by Lemar, Turner & Lloyd (2002), except that cells were embedded in Araldite resin not Spur. Essentially cells were fixed by suspension in buffered 1% osmium tetraoxide (OsO₄) in phosphate buffer (pH 7.2) for one hour at room temperature. The osmium fixed the lipids present in the bacterial membranes to make them visible by electron microscopy.

After fixation the cells were spun down in a microcentrifuge (Eppendorff 5414) for 2 minutes at 10,000 g and osmium tetraoxide was removed with a Pasteur pipette. This was then replaced with 30% ethanol, and incubated for 5 minutes at room temperature. The cells were then spun down again and the 30% ethanol removed. Cells were then coated in 3% agar (Agar Scientific, Cambridge, UK) using a cocktail stick to ensure that the whole pellet was covered, left to set for 5 minutes and excess agar was then trimmed to give just the pellet of cells bound together. The pellets were serially dehydrated by resuspension in 50% ethanol for 10 minutes. The 50% ethanol was then removed and pellets resuspended in 70% ethanol for ten minutes and the procedure was repeated for 80%, 90% and 100% ethanol solutions. If the cells could not be processed fully on the same day they were left in 70% ethanol and kept at 4°C overnight.

4.3.3 Transmission Electron Microscopy

After the ethanol replacement series to dehydrate the specimen, the pellet was placed into a fresh glass container with 100% ethanol for 15 minutes; it was then transferred to fresh 100% ethanol for a further 5 minutes. The pellets were then transferred to 5 ml propylene oxide which was very volatile for 15 minutes to displace any ethanol still present. The
pellets were then transferred to 50:50 propylene oxide/Araldite resin (5 g araldite CY212 and 5 g DDSA were preheated, mixed together and added to 0.3 g of BDMA, (Agar Scientific, Cambridge, UK). About 9 ml of this mixture was added to each sample and left over night in a rotary mixer (Agar Scientific, Cambridge, UK); the mixer rotated the samples at an angle that aided infiltration of resin into the pellet and evaporation of the propylene oxide.

The infiltrated pellets were transferred into 5 ml fresh resin (without propylene oxide) and left in the rotary mixer for a further 12 hours. Once the incubation was completed the pellets were transferred into moulds previously filled with full strength resin and pushed to the very bottom with a cocktail stick; these were left in the oven at 60°C for 48 hours.

Before sectioning the pellets were trimmed to remove excess resin using a razor blade. The ultra thin sections were then cut using a diamond knife and an ultratome III (LKB, Stolholm, Sweden). Once the ultra thin sections were cut they were placed on piloform coated 3.00 nm copper grids (Agar Scientific, Cambridge, UK).

Staining was carried out by placing a drop of 2% uranyl acetate from a pasteur pipette onto Nesco film, and floating grids section side down, in the stain, before incubating for ten minutes at room temperature in the dark. Then the grids were similarly transferred to a drop of lead citrate and left to incubate for 5 minutes. The grids were then washed three times in the same manner as above in filter sterilised distilled water.

For the TEM, 21 blocks containing EMRSA-15 were prepared and at least 4 grids from each of the 21 blocks were stained and used for the examination. The microscopy was
performed using a 1210 Joel transmission electron microscope. The electron micrographs were examined for structural differences between control and treated cells, and the presence of whole, partial or no septa was recorded. More than 1000 cells for each specimen were observed and examined for either the presence or absence of septa. The percentage of cells containing either partial (presence of septa at any stage of formation) or complete septa was calculated. The results were analysed statistically for significance using the ANOVA test in Mini Tab Statistical package (version 14).

4.4 Results

4.4.1 The effect of manuka honey on the structure of MRSA

For each time point 6 sections were cut and a minimum of 9 TEM micrographs were taken for each sample at every time point. The percentage of cells with septal components seen in all samples at time 0 varied between 35 – 50%. In the untreated cells the percentage with septal components remained between 35 -50% throughout the time course and visually it could be seen that the cells were entire with no blebs or lesions and cells without any septal components were clearly seen. The thin sections that contained cells treated with concentrations of manuka honey above the MIC, of 3% v/v for MRSA (Cooper et al, 2002) showed clearly that a greater proportion of cells possessed partial or complete septa compared to control cells (Figure 19). Those cells treated with less than 3% (v/v) manuka honey or with 10% (w/v) artificial honey did not show any significant difference in septa formation from control cells. In all samples examined the majority of the cells were entire, the cell surface did not appear to be damaged and evidence of cellular lysis was absent. Compared to the untreated cells the numbers of septal components in cells treated with
bactericidal concentrations of manuka honey (5, 10 and 20 % w/v) were significantly different with p = < 0.001 using a one way ANOVA. However those cells treated with the sub-inhibitory concentration of manuka (2.5% w/v) and 10% (w/v) artificial honey were not significantly different from the untreated control cells (p = 0.09 and p = 0.098 respectively).
Figure 19: The effect of varying concentrations of manuka honey on the percentage of cells showing septal components: Percentage of MRSA cells with septal components (partial and complete septa) following exposure to manuka honey. EMRSA-15 NCTC 13142 was incubated in nutrient broth (NB) (open triangle), NB with 10 % (w/v) artificial honey (open circles), NB with 2.5 % (w/v) manuka honey (open squares), NB with 5 % (w/v) manuka honey (filled triangles), NB with 10 % (w/v) manuka honey (filled diamonds), NB with 20 % (w/v) manuka honey (filled squares).
The increased numbers of septa seen after 60 minutes in those cells treated with honey above the bactericidal level did not rise further with time. Similarly, boosting the concentration of honey above the bactericidal level did not amplify the effect and 5% (w/v) manuka honey seemed to cause an equivalent effect as the 20% (w/v) manuka honey (Figure 19). This shows that the effect is not modulated by dose or time of exposure. This is in accord with the data from the time kill experiments (p52) which indicated no increased response once the MIC/MBC had been exceeded.

Control cells (Figure 20: a, b and c) appeared to be of normal size and morphology, those cells treated with bactericidal levels of manuka honey (Figure 20: d, e and f) seemed to have a slightly enlarged appearance when compared to control cells. The TEM micrographs presented here have been chosen to illustrate the changes seen in cells at T120 as they are representative of cells treated with honey and no further distinct changes visible up to 24 hours; they are just a small selection of the many micrographs taken.
Figure 20: Electron micrographs of untreated and manuka honey treated EMRSA-15:
The effect of manuka honey on the structure of EMRSA-15, NCTC 13142 cells. Electron micrographs show MRSA at 32,000 magnification following incubation for 120 minutes (a) in nutrient broth (NB) alone, (b) in NB containing 10% (w/v) artificial honey, (c) in NB containing 2.5% (w/v) manuka honey, (d) in NB containing 5% (w/v) manuka honey, (e) in NB containing 10% (w/v) manuka honey, (f) in NB containing 20% (w/v) manuka honey. The number of cells with septa is markedly increased following treatment with bactericidal concentrations of manuka honey (above 3% w/v).
4.5 Discussion

This study demonstrates that the treatment of MRSA with inhibitory concentrations of manuka honey has an effect on the ability of the MRSA to complete the cell cycle. Furthermore it has been shown by the appearance of the cells that this effect is not due to the presence of sugars in the honey as treatment with 10% (w/v) artificial honey did not produce the same effect as treatment with 10% (w/v) manuka honey.

Many antimicrobial agents, both traditional ones such as essential oils of oregano or rosewood and more mainstream antimicrobials such as quinupristin have been shown by electron microscopy to cause cell wall damage which leads to cell lysis (Castillo et al 2006; Carson et al, 2002; Lorian and Fernandes, 1999). However, the absence of visibly lysed cells and the lack of cellular debris in electron micrographs of cells exposed to inhibitory concentrations of manuka honey used in this study suggest that the prime target site in MRSA is unlikely to be associated with cellular functions that alter the integrity of the cell wall itself.

The electron micrographs suggest that although MRSA cells treated with bactericidal levels of manuka honey were capable of forming a septum prior to cell division, they did not seem capable of completing the process of cell division because replicated cells remained undivided, apparently stuck at the point of cell separation immediately prior to the completion of the cell cycle. This effect was initiated very rapidly within 30 minutes of exposure to inhibitory concentrations of manuka honey and the increased percentage of cells with septal components remained consistent between 1 and 24 hours of treatment. It was also clear that a relatively small increase in concentration of manuka honey above the
MIC of 3 % (v/v) was required to elicit this effect and that further increases in concentration did not lead to additional increases in the number of cells with completed septa. The rapid response seen could be due to the honey altering the protein expression or distribution within the cells at a critical point (cytokinesis) and therefore very quickly stopping the cells from progressing through a normal cell cycle.

These observations suggest that the inability of cells to divide and separate may be due to an effect on either the expression or recruitment of cell division proteins such as FtsZ or the twelve or so essential division proteins that associate with this initiator of cell division forming a scaffold for construction and constriction of the Z ring (Addinall and Holland, 2002). Additionally a change may be induced in the quantity or distribution of enzymes involved in cell separation and hydrolysis of cell wall, such as the murein hydrolases that are recruited after the formation of septum and are required to digest the peptidoglycan bonds holding the two daughter cells together. It has been shown previously that disruption of the autolysins in *S. aureus* can lead to abnormalities in cell wall formation and eventually to lysis (Sugai *et al*, 1997; Yamada *et al*, 2001).

The apparent increase in cell size was not measured but could indicate that growth continued after the induction of the defect(s) that led to the failure of the cell to separate. Any such growth is unlikely to have continued for long, as no formation of giant cells or additional evidence of multiple septum formation was observed within the enlarged, undivided cells. The presence of enlarged cells with completed septa, however, could indicate that cell growth may have continued even though the septum had formed at the correct time but cells did not separate. The presence of viable cells after 3 hours exposure to an inhibitory concentration of manuka honey 10% (w/v) as seen by confocal microscopy
despite decreasing numbers determined by total viable cell count supports this hypothesis. If the septum had been formed prematurely it is unlikely that two cells of enlarged size would have been seen, so the inhibitory effect is probably not associated with the early triggering of septum formation.

This study does demonstrate that incubation of EMRSA-15 with bactericidal levels of manuka honey has an effect on the ability of the cells to complete the cell cycle in *vitro* showing significantly more cells with septal components (*p* = <0.001) and offers a potential explanation of the mechanisms by which manuka honey could be exerting its known therapeutic effects. Therefore there was a need to investigate whether this effect caused a problem in formation of the septum by division proteins such as FtsZ or whether the effects were after this step altering the integrity of the separation mechanisms.

This analysis led to the use of 10% (w/v) manuka honey throughout the rest of the study instead of a range of concentrations, as it caused a significant effect on the cell cycle.
5. Cell separation - A Study of the Change in Murein Hydrolase Profile in Bacterial Cells Treated with Manuka Honey (Meticillin Resistant *Staphylococcus aureus*)

5.1 Introduction

The cell wall of *Staphylococcus aureus* is composed of a wide range of components, all of which serve different functions. Many of these functions are essential to maintain cell viability. The primary function of the cell wall is to provide the cell with an exoskeleton structure that defines the cell shape and protects the bacteria from lysis, however many of the proteins found within the cell wall are involved in survival within a host or in controlling synthesis and turnover of the cell wall and cytokinesis (Navarre and Schneewind, 1999).

Although a detailed understanding of the precise events of the cell cycle of staphylococci is not yet available, cell division is thought to be controlled in part by murein hydrolases (Holije and Tuomanen, 1991; Takahashi *et al*, 2002; Biswas *et al*, 2006), which are a diverse family of enzymes designed to cleave the structural components of the cell wall and, therefore, critical to normal cell growth and division. Aside from cleaving peptidoglycan at cell division, murein hydrolases have a large range of functions (Table 9 adapted from Vollmer *et al*, 2008). These enzymes include: N-acetylmuramidase, N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase and endotransglycosidase activities.
**Murein Hydrolase functions** | **Explanation** | **Examples**
--- | --- | ---
**Cell Physiology** |  |  |
Regulation of cell wall growth | Removes excess pentapeptides in new peptidoglycan | DDcarboxypeptidase, PBP3 *Streptococcus pneumoniae*
Enlargement of sacculus | Breaks bonds allowing expansion | Lyt E, *B. subtilis*
Peptidoglycan turnover | Release of soluble peptidoglycan from sacculus during growth | Lytic transglycosylases, *Escherichia coli*
Production of signalling molecules | Induces β-lactam by peptidoglycan turnover products | Lytic transglycosylases, *E.coli*
Recycling of peptidoglycan turnover products | Cleavage of products for reuse in peptidoglycan synthesis | AmpD, *E. coli*
Cell separation during/after division | Cleavage of cross wall (septum) | Atl, *S.aureus*
Sporulation and Germination | Cleavage of asymmetric septum, Spore cortex maturation, Peptidoglycan digestion to release spore | Lyt H, *Bacillus subtilis*
Assembly of secretion systems | Localised peptidoglycan degradation associated with secretion systems types II, III and IV | VirB1 *Agrobacterium tumefaciens*
Pilus assembly | Peptidoglycan hydrolases for assembly | PilT, *E. coli*
Flagellum assembly | Peptidoglycan hydrolases for assembly | FlgJ, *E. coli*
Resuscitation of dormant cells | Stimulation of cell division | RpfA, *Micrococcus lysodeiktikus*

**Functions within bacterial populations**
|  |  |  |
Developmental lysis | Cannibalism, Lysis of cells during fruiting body formation | *B. subtilis, S. pneumoniae*
Lysis of prey | Digestion of prey peptidoglycan | Exoenzymes, *Myxococcus xanthus*
Autolysis in genetic transformation | Practicide, induced lysis of non competent cells | LytA, LytC, *S. pneumoniae*
Lysis of non immune cells | Plasmid encoded bacteriocin | Pesticin, *Yersinia pestis*
Biofilm formation | Initial attachment of cells to hydrophobic surfaces | AtlE, *Staphylococcus epidermidis*

**Table 9: Functions of bacterial peptidoglycan hydrolases:** Shows multiple functions of bacterial murein hydrolases in a number of species. Adapted from Vollmer et al, (2008)
The cell wall in *S. aureus* is constructed of glycan chains. These are comprised of alternating subunits, N - acetylglucosamine (GlcNAc) and N - acetylmuramic acid (MurNAc) which are cross linked by bridges involving the lactyl moiety of MurNAc (Figure 21). This arrangement creates a mesh like structure which is continually expanded as the cell grows (Dmitriev *et al*, 2004; Dmitriev *et al*, 2005; Scheurwater *et al*, 2008; Vollmer *et al*, 2008b). It is the links between these glycan chains that are targeted by the murein hydrolases, however these enzymes have high specificity and each murein hydrolase targets a particular site.

There are many types of murein hydrolases that have been identified and these are divided into two classes. Class 1, are those that are encoded on the bacterial chromosome, exported by N terminal signal peptides and usually involved in cell wall turnover; and class 2, those encoded on bacteriophage chromosome and which have no N terminal signal peptide. These are exported by a more general lysis mechanism. These hydrolases can be further divided by grouping according to their enzymatic activity for example: N-acetylmuramidase (muramidase), N-acetylglucosaminidase, Amidase, Lysostaphin and hydrolase produced by phage 11. The sites at which these enzymes cleave the cell wall can be seen in Figure 22 (Navarre and Schneewind, 1999).

The ability of these enzymes to potentially lethally hydrolyze the cell wall means that they are under tightly regulated control at both transcriptional and posttranslational levels by several ‘two-component’ regulatory systems, accessory gene regulators and other components including *LrgAB, CidAB*, rat and Gcp. In particular Gcp has recently been shown to be essential for bacterial survival (Zheng *et al*, 2005) and affects several of the other regulators; therefore it is a potential target site for the honey.
It has been reported that deficiency in murein hydrolases can lead to failure of the cell to separate (Rice et al., 2003; Yamada et al., 1996; Sugai et al., 1995; Yamada et al., 2001).

**Figure 21: Peptidoglycan Cell Wall Structure:** Peptidoglycan structure found in the *Staphylococcus aureus* cell wall. Short wall peptides bind GN (N acetyl glucosamine) and MN (N acetyl muramic acid) through the lactyl moiety in MurNAc. Cross bridges link adjacent wall peptides (Vollmer et al., 2008).
Hydrolytic activity in *S. aureus* has previously been detected in both extracellular fractions and also in broken cell wall and intracellular fractions of cells (Tipper, 1968; Singer *et al.*, 1972). This meant it was important to investigate potential changes in both intracellular and extracellular fractions induced by treatment with honey. It has been shown that treatments can affect hydrolases in varying ways, higher rates of overall autolysis can be achieved while at the same time cell wall bound hydrolases are inhibited (Groicher *et al.*, 2000). Therefore there could be an effect on autolysin production that is universal for all

Figure 22: Peptidoglycan hydrolase sites of action The sites of action of several murein hydrolases, Glucosaminidase is highlighted as β-N-acetylglucosaminidase is studied in more detail in Chapter 6. Adapted from Navarre and Schneewind, (1999).
autolysins produced by the cell. Analysing both fractions allows not only the rate of activity to be measured but also gives an insight into potential alterations of targeting and secretion of these enzymes.

5.2 Aim

The aim of this chapter was to investigate changes in murein hydrolase function in MRSA in response to exposure to 10% (w/v) of manuka honey for up to 24 hours, using enzyme assays and zymography.

5.3 Methods

From overnight broth cultures, 30 ml of EMRSA-15 were used to inoculate flasks containing 270 ml tryptic soy broth alone (TSB) (Oxoid), or TSB with 10% (w/v) manuka honey or 10% (w/v) artificial honey, giving a final volume of 300 ml. Samples were then removed at 0, 30, 60, 90, 120, 240 and 1440 minutes for both cell wall assays and zymography in intracellular and extracellular fractions. Sterile gamma irradiated honey was provided by Advancis Medical, phenol equivalent was 18. For every sample collected both intracellular and extracellular preparations were made. Each extract from each time point was tested in triplicate by both assay and gel methods.

5.3.1 Collection of Intracellular and Extracellular Fractions Containing Hydrolase Enzymes

The culture supernatant was harvested by centrifuging the cultures (300 ml) for 20 minutes at 6000 g at 4°C (Sorvell RC5B, DuPont Instruments). It was then concentrated approximately one hundred-fold using a centricon PL-10 concentrator (Millipore, Bedford,
Mass) according to manufacturer’s instructions and stored at -20°C. The intracellular proteins were extracted from the pellet remaining after the initial centrifugation step by resuspending in 20 ml phosphate buffered saline and the cells were treated in a French press at 3000 lbs p.s.i. The crude extract was collected and then centrifuged at 3000 g for 30 minutes at 4°C to remove any remaining whole cells or debris. The supernatant was stored at -20°C. Protein concentrations of each of these fractions were determined using the Bradford method (Bio-Rad laboratories, Hercules, California) according to the manufacturer’s instructions.

5.3.2 Murein hydrolase Cell Wall Assay

Murein hydrolase activity was estimated using cell wall assays performed as described by Mani et al., (1994). Briefly, 100 µg of enzyme extract was added to a suspension of autoclaved and lyophilized Micrococcus lysodeikticus cells (1.0 mg/ml) (Sigma, Poole, Dorset) in 100 mM Tris HCl (pH 8) and incubated at 37°C with shaking at 120 rpm. Turbidity measurements were taken at 30 minute intervals using a Cecil spectrophotometer at 580 nm using TSB as a blank. This assay was performed in triplicate for every extract prepared. Protein concentrations of each of these fractions were determined using the Bradford method (Bio-Rad laboratories, Hercules, California) according to the manufacturer’s instructions.

5.3.3 Zymography

Equal amounts of protein (15 µg) each of intracellular and extracellular autolysin fractions were electrophoresed in SDS-polyacrylamide gel (15%) containing 1% w/v M. lysodeikticus cell walls (Sigma) (Leclerc and Asselin, 1989). Lysostaphin was also run in
one lane as a control along with Colorburst electrophoresis size standards in another lane (Bio-Rad, Hemel Hempstead, UK). These were chosen as they were the only size standards still visible after staining of the gel. Following electrophoresis, the gel was incubated in renaturation buffer (25 mM Tris-HCl pH8) containing Triton X-100 overnight at 37°C. The gel was then stained using 0.01% (w/v) methylene blue in 0.1% (w/v) potassium hydroxide as used in Strating and Clarke (2001). The gel was destained with deionised water. Gels were run in triplicate and photographed using the UVP GelDoc, Bioimaging System, AutoChemi, Cambridge). Hydrolase activity was observed as clear substantial regions in the gel.

5.4 Results

5.4.1 Murein Hydrolase Cell Wall Assay

In this assay enzyme activity was expressed as a percentage of the turbidity observed at time 0, so that if hydrolase enzymes are present and active the turbidity decreased due to lysis of the cell walls present (Mani et al., 1994). It is normal, therefore, to expect a drop in turbidity with time, in this experiment however activity was essentially only seen in those intracellular fractions of cells cultivated for 1440 minutes in either TSB alone (control cells), or those treated for 1440 minutes in TSB plus 10 % (w/v) artificial honey (Fig. 18).

The cell wall assays showed a significant reduction in the amount of intracellular murein hydrolase activity produced in those cells treated with manuka honey after 1440 minutes when compared to untreated control cells p = <0.0001 (Figure 23). Greatest activity was seen in those cells treated with 10 % (w/v) artificial honey for 1440 minutes. This treatment
caused the opposite effect to manuka honey and significantly increased murein hydrolase activity compared to the control cells after incubation for 1440 minutes (p = 0.025). These tests were done using a one way ANOVA in Microsoft Minitab version 14. There was no visible activity seen with any of the extra cellular extracts for any treatment at any time point. There was also no visible effect seen on intracellular extracts at any other time point, this could be due to no enzyme being present or the method not being sensitive enough to pick up the lower activity at earlier time points.
Figure 23: Murein hydrolase cell wall assay using intracellular and extracellular extracts from EMRSA-15 treated with and with honey: The effect of manuka honey on murein hydrolase activity. The murein hydrolase activity was then determined in EMRSA-15 that had been incubated for 24 hours in nutrient broth (TSB) alone, or in TSB with 10% (w/v) artificial honey or in TSB containing 10% (w/v) manuka honey. The intracellular and extracellular proteins were then isolated and hydrolase activity determined in the assay above. The assays were performed in triplicate and mean relative activity is presented ± standard deviation.
5.4.2 Zymography

The pattern of banding seen on the zymographs corresponded with the results obtained in the murein hydrolase cell wall assays. There was a clear difference between the numbers of bands in the intracellular extract of MRSA exposed to 10 % (w/v) manuka honey for 1440 minutes when compared to untreated cultures at the same time point. Intracellular extracts of MRSA exposed to 10 % (w/v) manuka honey had markedly fewer bands in zymographs than untreated cells, yet those of artificial honey treated cells demonstrated additional bands (Figure 24). Again no effects were seen on any of the other intracellular extracts at different time points and no difference was seen in any of the extra cellular extracts compared to the control cell extracts.

Zymography was also carried out using a gel containing 1 % (w/v) freeze dried S. aureus cell walls (Sigma) instead of the M. lysodeiktikus, however this did not disperse evenly within the gel, therefore M. lysodeiktikus was used as it gave clearer banding patterns.
Figure 24: Zymograph of intracellular and extracellular extracts of EMRSA-15 after 1440 minutes treatment with and without honey: Zymograph showing inhibition of murein hydrolase production in intracellular extracts treated with honey for 24 hours. Intracellular extracts from untreated cells (lane A), cells exposed to 10 % (w/v) artificial honey (lane B) and 10 % (w/v) manuka honey (lane C); extracellular extracts from untreated cells (lane D), cells exposed to 10 % (w/v) artificial honey (lane E) and 10 % (w/v) manuka honey (lane F). Lysostaphin was in lane G and Colorburst™ electrophoresis size standards (Bio-Rad, Hemel Hempstead, UK) in lane H.

5.5 Discussion

This set of experiments demonstrates that the treatment of MRSA with an inhibitory concentration of manuka honey (10% w/v) had a significant effect on the production of the hydrolytic enzymes that are necessary for correct completion of the cell cycle. Furthermore it was clear that this effect was not due simply to the sugars in the honey as the artificial honey elicited quite separate and opposite effects on murein hydrolase profiles.
Many antimicrobials cause cell wall damage which leads to cell lysis (Sawer et al., 2005). Manuka honey has previously been shown to impede cell growth at cell division which suggests that the primary mode of action is not cell wall damage (Henriques, 2006). Data from this experiment suggests that that the failure of the cells to grow and divide properly after treatment with manuka honey could be due to a change in the amount or distribution of cell division enzymes produced in the cells after treatment with honey above the MIC. The murein hydrolase assays showed that there was a marked reduction in the amount of intracellular murein hydrolase activity produced by the cells treated with manuka honey, but conversely a significantly increased production by those cells treated with artificial honey. This indicates that manuka honey contains an unknown component that has the ability to influence activity or production of hydrolase enzymes which could in turn impede cell division (Rice et al., 2005).

The changes in hydrolase profiles deduced from the murein hydrolase cell wall assay were also reflected in the digestion patterns on the zymographs (Figure 24), a reduced effect was seen from honey treated cells (lane 3) after 1440 minutes, whereas the banding patterns seen for control and artificial honey treated cells (lanes 1 and 2 respectively) were similar, with the artificially treated extract even appearing to produce an extra band of digestion.

As well as murein hydrolases, it is possible that other factors involved in the process of cell division are being affected by manuka honey treatment; the delay or halt in the cell cycle could be regarded initially as a sub lethal injury leading to irreversible damage to the cell.
Cell division is a highly complex process that has not been fully elucidated, involving numerous proteins and enzymes. The daughter cells are formed after the newly formed septum is cleaved by these enzymes (Errington et al, 2003). The regulation of these enzymes is not fully documented but some components are known (Bayles, 2000) therefore the results observed during this investigation could be due to premature formation of septa without other essential cellular events having taken place. There could be an effect on the structural division proteins (such as FtsZ) or on the target sites of the bacteriolytic enzymes involved in the cleavage of peptidoglycan. Alteration in the specific binding sites would mean that enzymes would not recognise and therefore not cleave structural components.

It can be difficult to assign specific functions to all of the peptidoglycan hydrolases found in *S. aureus*, or in any other species of bacteria as there are many of them. Each with overlapping or multiple functions, so that the loss of one enzyme does not necessarily mean loss of a activity. The reduced activity of these peptidoglycan hydrolases might also be due to inability to target the substrate. Commonly conserved repeat motifs which create ionic attachment to peptidoglycan are found on these enzymes and modification of these sequences could lead to an inability to find the correct target.

Previous zymography work has shown that a normal hydrolase profile of *S. aureus* can have 6 bands of digestion (Ledala et al, 2006). Other work has shown in turn that zymographs from inactivated *atl* mutants have only one band of digestion demonstrating that there are other murein hydrolases important in *S. aureus*. An investigation conducted by Rice and colleagues (2005) reported 34, 50 and 110 kDa murein hydrolase bands on gels which could be some of the network of hydrolases that exist in *S. aureus* and which have complex control mechanisms regulating hydrolase activity. Any of the components of this
network if affected by the honey treatment could lead to an alteration in the murein hydrolase profiles seen. Figure 25 has been compiled from what is known about the various regulatory components of murein hydrolases.

The two component regulatory system \textit{LrgAB} causes decreased murein hydrolase activity in the extra cellular fraction, suggesting that it has a function analogous to bacteriophage encoded antiholins (Rice \textit{et al}, 2004). Antiholins are normally released from bacteriophages, they are small inhibitory proteins that prevent the function of holins, reducing the access of cell wall hydrolytic enzymes to the cell wall substrate (Blasi and Young, 2003). The \textit{LrgAB} system is directly downstream of two component regulatory system \textit{LytSR}, which transcriptionally activates \textit{LrgAB} and it is also downstream of two component regulatory systems \textit{AgrCA} (accessory gene regulator) and \textit{ArlRS} which positively regulate the operon, these three regulators have membrane sensors and response regulators (Fournier and Hooper, 2000; Groicher \textit{et al}, 2000; Rice \textit{et al}, 2006).

The \textit{LrgAB} system is also downstream of SarA (staphylococcal accessory regulator) a ‘one-component’ regulatory system that also positively regulates expression of anti holins. \textit{Lrg} operon has two overlapping transcripts \textit{lrgA} and \textit{lrgAB}. Open reading frames seem to be translationally coupled, 5’ ends of \textit{lrgB} and \textit{cidB} ORF overlap 3’ ends of \textit{lrgA} and \textit{cidA}. Both \textit{Agr} and \textit{Sar} are interactive global regulators regulating the biosynthesis of virulence factors, Sar A upregulating \textit{σB} involved in both stress response and virulence factor production (Chan \textit{et al}, 1998).
Figure 25: Control of murein hydrolase expression in *S. aureus*. Murein hydrolase expression has several points of control. Control of the major autolysin (Atl) expression and activity does not appear to be directly related to these control mechanisms. 


All of those regulators are in turn controlled by the expression of *Rat*, an auto regulating regulator that modulates the expression of the regulators downstream of it. It also negatively regulates the expression of *LytM/N* which produces glycylglycine endopeptidase and cell wall hydrolase respectively. It may regulate *LytM/N* indirectly via regulators like
Lyt SR but it could also bind directly to these target genes. It is thought that it also has a role in regulating the expression of abcA, scdA, sspA genes that are thought to have an effect on autolytic activity (Ingavale et al., 2003).

Alongside the expression of lrgAB is a cidABC which is homologous to lrgAB but with the opposite effect, producing a holin type effect, promoting access of murein hydrolases to the peptidoglycan substrate (Lossner et al., 1999; Patton et al., 2006), therefore increasing murein hydrolase activity in the extra cellular fraction. Holins are proteins which once inserted into the membrane cause lesions, these lesions allow enzymes access to peptidoglycan by forming channels across the cytoplasmic membrane, and therefore are related to the timing of lysis within bacteria (Young, 1992; Takac et al., 2005). It has two overlapping transcripts cidAB and cidBC. It is positively regulated by transcriptional regulator cidR and sigma factor β (which also down regulates lrgAB production). Both of those regulators are in turn regulated by rsbU gene that modulates their activity (Rice and Firek, 2003). Expression of major atl is not directly linked to any of the above regulators and the systems by which it is controlled are not well elucidated but as it is the main hydrolase and there was a large reduction in hydrolase activity it seems very likely that the honey is affecting this enzyme synthesis in some way.

The decrease in hydrolase activity could also indicate an effect on Gcp as previous studies have shown that down regulation of this glycoprotease leads to decreased autolytic activity due to Gcp normally functioning by positively regulating cidAB operon and negatively regulating lrgAB operon (Zheng et al., 2007). Equally the effects seen in the above experiments could be due to aberrations in any of the translational or posttranslational modifications of these enzymes or even in the chemical composition of the cell wall with
cell wall polymers such as lipoteichoic acids being known to inhibit hydrolase activity (Vollmer et al, 2008). Interestingly growth of bacterial species at low pH has been shown to reduce bacteriolysis so the acidic pH of the honey may well be directly contributing to the reduction of peptidoglycan hydrolase activity seen.

The present study has demonstrated that incubation of MRSA with bactericidal levels of manuka honey has a marked effect on the ability of the cells to complete the cell cycle and offers a potential explanation of the mechanisms by which manuka honey exerts its known therapeutic effects. This effect is not attributable to the presence of the major sugars contained in honey, as shown by the results from the cells exposed to artificial honey in this chapter. The precise effect of manuka honey on the synthesis and regulation of murein hydrolases and other enzymes and proteins involved in cell division is the subject of other chapters.
6. The Effect of Manuka Honey on FtsZ and Endo – β – N - Acetylglucosaminidase in EMRSA-15

6.1 Introduction

Cell division is an essential process for the life cycle of bacteria; improved understanding of the mechanisms of cell division in *S. aureus* could improve the discovery and design of novel antibacterial agents to target this organism (Datta *et al.*, 2006). For staphylococci to survive it is essential to segregate genetic material into two equal parts prior to cell division (Corbin *et al.*, 2007). The earliest stage of cell division is the formation of the Z ring around the inner surface of the cytoplasmic membrane; the first components of this process to localize to the future division site are the FtsZ polymers (Blaauwen *et al.*, 1999).

FtsZ is a GTPase and has been identified as an essential constituent of cell division. An ancestral homologue of tubulin, it is the earliest gene product required to initiate this process and is recognised as an indicator of division initiation.

It has an N terminal domain made of a six strand B-sheet surrounded by several alpha helixes (Figure 26). FtsZ localises to midcell, the site of future cell division, forming protofilaments in the presence of GTP to create the Z ring. Z ring formation and consequent division of the cell is a dynamic process involving several other proteins recruited in a linear hierarchy, although it is thought that multiple interactions between these proteins occurs outside the linear order of dependency. Localization of proteins to the FtsZ framework leads to the formation of two daughter cells (Weiss, 2004; Harry, 2001; Errington *et al.*, 2003; Bramhill, 1997).
Figure 26: Ribbon drawings of FtsZ (residues 23–356) from Methanococcus jannaschii. View showing the GTPase domain in blue/green, the C-terminal domain in red/orange, and the connecting helix H5 in yellow. GDP is represented by pink and blue spheres (Taken with permission from: Lowe and Amos, 1998).
Once the ring is formed GTP in conjunction with FtsZ causes a shift in the structure of FtsZ from straight to curved form, possibly driving constriction of the septum at division (Yan et al., 2001; Lu et al., 2000). There are two models for the possible action during constriction of FtsZ: these are sliding protofilament (Fig 27) and depolymerisation (Figure 28)(Errington et al., 2003).

The fact that FtsZ is highly conserved between species of bacteria and found in nearly all eubacteria and archaea makes it a highly attractive candidate as a future therapeutic target (Harry et al., 2006; Margolin, 2000; Addinall and Holland, 2002).

In view of the results presented above (chapter 4) which indicate interference with cell division by manuka honey, it is clear that FtsZ is also a potential candidate target site for the action of honey on MRSA.
Figure 27: Illustration of FtsZ sliding protofilament model of constriction. FtsZ polyfilaments slide over one another driven by a motor causing bending of filaments and constriction of the membrane (adapted from Bramhill, 1997).
Figure 28: Illustration of FtsZ depolymerisation model of constriction. FtsZ constriction driven by loss of FtsZ subunits from the end of the polymer at junction with an anchor (adapted from Bramhill, 1997).
The cell wall turnover of *S. aureus* is dependant upon three distinct biochemical stages that occur in the cytoplasm, in the membrane and in the wall itself. Cell wall assembly and modification critical to cell division is catalyzed by penicillin binding proteins (PBPs) that cause polymerisation and transpeptidation of wall peptides giving a distinctive cell wall structure (Navarre and Schneewind, 1999). For the cell to complete cell division and form daughter cells, this structure must be hydrolyzed at the correct points by specific enzymes in a controlled manner. Peptidoglycan hydrolases (also known as murein hydrolases) are a group of enzymes involved in degradation of the links within the peptidoglycan polymer that is found in the cell wall material of *S. aureus*.

The enzyme products of the *atl* gene in *S. aureus* are thought to play a major role in the events of the cell cycle because this gene encodes *S. aureus* major Atl and produces two predominant peptidoglycan hydrolases, which are tandemly encoded in a single open reading frame (*atl*). The gene product is a bifunctional 138kDa protein (1256 amino acids) that splits to become 51kDa endo-B-N-acetylglucosaminidase (GL) and 62kDa N-acetylmuramyl-L-alaninamidase (AM). The AM domain extends from bp 2588 to bp 4300 with corresponding Atl polypeptide extending from Ala199 to Lys775.

The GM domain extends from bp 4301 to bp 5743 with corresponding Atl polypeptide extending from Ala776 to Lys1256 (Baba and Schneewind, 1998; Oshida and Sugai, 1995). The control of these Atl enzymes is not well clarified but it does not seem to be directly related to any of the two component regulators that influence the production of other hydrolases in *S. aureus*, nor does it appear to be under downstream control of Gcp (glycoprotease) which again influences the production of other murein hydrolases (Zheng et al, 2005).
Defects in staphylococcal production of Atl (which splits into an amidase and glucosaminidase) have been shown in previous studies to lead to abnormal growth and division of the cells. Increased and decreased production of this enzyme has been shown to be caused by antibiotics currently in use. Decreased production has been shown to lead to clustering of the cells due to failure of the cell to hydrolyze the cell walls and split correctly (Foster, 1995; Ledala et al, 2006; Oshida et al, 1995; Yamada et al, 2001).

6.2 Aim

To investigate the effect of 10 % (w/v) manuka honey on the intracellular localization of FtsZ and Endo – β – N - Acetylglcosaminidase in MRSA using immunofluorescence and gold labelling. The regulation of expression of mRNA for these proteins was estimated by RT-PCR.

6.3 Methods

As in previous chapters the culture used in these experiments was EMRSA-15. Anti FtsZ raised in rabbits was kindly donated by Professor Jeff Errington of Newcastle University and anti Endo-B-N-Acetylglcosaminidase was used (Nordic immunological laboratories, The Netherlands) raised in rabbit.. Two secondary antibodies were used. During fluorescence microscopy anti rabbit FITC (Sigma, Poole, UK) was used and during immuno-localization by electron microscopy an anti rabbit coupled to 10 nm gold particles (Amersham and British Biocell) were used. Methods adapted from Harry et al, 1995; Blaauwen et al, 1999 and Pinho and Errington, 2003; Roth et al, 1978.
6.3.1 Localisation of FtsZ and Endo – β – N - Acetylglucosaminidase using Immunofluorescence

Overnight cultures of EMRSA-15 were inoculated into each of three flasks, one containing 100 ml TSB, one containing TSB plus 10 % (w/v) manuka honey and another containing TSB plus 10 % (w/v) artificial honey. After inoculation the flasks were incubated at 37°C in shaking water bath at 120 rpm and samples were removed at 0, 15, 30, 60, 90 120, 180, 240 and 1440 minutes. At each time point a 0.5 ml sample was removed from each flask, spun down at 10000 x g for 2 minutes (MSE Microfuge, Sanyo) and the pellet of cells was fixed using 2.7% paraformaldehyde, 0.005% glutaraldehyde in PBS.

The cells were then washed three times in PBS and resuspended in GTE (glucose 50 mM, Tris HCl 20 mM, pH 7, EDTA 10 mM). A gentle lysis was performed using lysostaphin (Sigma) added to a final concentration of 20 ng/ml. 10 µl of this bacterial suspension was added to each of 8 wells in a multiwell slide (MP Biomedicals, LLC, Germany) where the wells had been coated with polylsine 0.1 % (w/v) (Sigma) immediately prior to use, to promote attachment of cells.

The liquid phase was removed and discarded after 60 seconds and the wells were air dried. Each well was then washed twice with PBS, rehydrated with PBS and blocked using PBS containing 2 % BSA for 15 minutes. Liquid was removed and 10 µl of the primary antibody (anti FtsZ or anti Endo – β – N - Acetylglucosaminidase) diluted to 1/10000 with PBS was added to 7 of the eight wells in each slide, leaving the remaining well as a negative control to check for non-specific staining. The slide was then incubated at 4°C overnight. Slides were washed eight times with PBS, the secondary antibody was added to every well at a concentration of 3/1000 and incubated in the dark for two hours at room temperature before
slides were washed eight times with PBS. Vecta shield mounting media containing DAPI (Vector laboratories, Peterborough UK) was added to every well before a cover slip was placed onto the slide. Slides were then visualized on an Olympus BX-40 fluorescence microscope at 1000 times magnification using U-MF2 filter for visualizing DAPI staining and U-MNB for visualizing FITC staining. At least six photos were taken for each sample at each time point, images were observed using Improvision Volocity software. Green fluorescence indicated the presence of FtsZ and this was counted in relation to the number of cells present which were visualised by DAPI staining nuclear material blue. Statistical analysis was done using Two way T test in Microsoft Minitab version 14.

6.3.2 Gold Labelling Localisation of FtsZ and Endo–β–N-Acetylglucosaminidase using Transmission Electron Microscopy of Whole Cells

A single colony from a 24 hr plate culture was grown overnight in a 20 ml volume of sterile nutrient broth (Oxoid) in a shaking (120 cycles/minute) water bath at 37°C. The total volume of cells were spun down at 3000 g for 30 minutes (MSE Harrier 15/80 centrifuge, Sanyo) and inoculum was resuspended in 20 ml of tryptic soy broth (Oxoid) containing 10% w/v manuka honey, or TSB with 10% w/v artificial honey or in tryptic soy broth alone. These 3 cultures were grown at 37°C in a shaking water bath for 24 hours. After growth 1 ml samples of each culture in eppendorf tubes were harvested at 10000 g for two minutes (MSE Microfuge, Sanyo). Once a pellet had formed a few drops of 5% glutaraldehyde (Sigma) was added to each eppendorf tube and incubated at 4°C for one hour. After fixation cells were washed in PBS containing 0.2% (w/v) BSA three times for
10 minutes, they were then incubated with 50 µl primary antibody 1/10000 (anti FtsZ or anti Endo – β – N - Acetylglucosaminidase ) for one hour, cells were washed again in PBS containing 0.2% BSA three times for 10 minutes each. Cells were then incubated with gold labeled with 50 µl secondary antibody diluted to 1:50 with PBS for 30 minutes before a final wash with PBS containing BSA. For each sample 2 µl of cells was transferred to 6 nickel grids and left to dry at room temperature. Microscopy was done using a 1210; Joel transmission electron microscope. At least ten pictures of each sample were taken.

This experiment was conducted at EM unit of Cardiff University with the help of Dr Hann.

6.3.3 Gold Labelling Localisation of FtsZ and
Endo – β – N - Acetylglucosaminidase using Transmission Electron Microscopy of Thin Sections

A single colony from a 24 hr plate culture was grown overnight in a 20 ml volume of sterile nutrient broth (Oxoid) in a shaking (120 cycles/min) water bath at 37°C. The total volume of cells were spun down at 3000 g for 30 minutes and inoculum was resuspended in 20 ml of TSB containing 10% w/v manuka honey, or TSB with 10% w/v artificial honey or untreated Tryptic soy broth. These were cultivated at 37°C in 120 rpm water bath for 24 hours. After growth 1 ml of samples were then harvested at 10000 x g for two minutes as before at room temperature. Each pellet was then fixed in 0.25% osmium tetroxide and 2% glutaraldehyde for half an hour. Cells were then washed twice in PBS and then coated in molten 6% agar (Agar Scientific, Cambridge, UK) to keep cells together. Cells were dehydrated in series from 30% ethanol to 100% ethanol, 30 minutes incubation for each step; this was done inside the automated freeze substitution system (Reichert AFS, Leica, UK). Once cells had been dehydrated they were placed in 1:1 Lowicryl HM20: Ethyl
alcohol (ETOH), to infiltrate pellet with resin and remove air bubbles, followed by 2:1 HM20 : ETOH, then embedded in HM20 and left at -30°C over night before being exposed to UV light for 2 days, to set the lowicryl resin.

The ultra thin sections were then cut using a diamond knife and an ultratome III (LKB, Sweden). Once the ultra thin sections were cut they were placed on uncoated 3.00 nm nickel grids (Agar Scientific). The sections were then incubated with 5% sodium metaperoxide for 10 minutes to remove osmium tetroxide. The sections were blocked overnight at 4°C using 2% Tris buffered saline containing 0.2% BSA and 0.1% sodium azide, pH 7.6. Grids were then exposed to primary antibody anti FtsZ or Endo – β – N - Acetylglucosaminidase raised in rabbit that was diluted 1:10000 with PBS overnight at 4°C. Grids were washed six times in Tris buffered saline before being left overnight at 4°C in secondary antibody diluted to 1:50 with PBS, anti rabbit coupled to gold (Amersham and Biocell 10 nm) at a concentration of 1:20. Negative controls of each sample (control, artificial and honey) were also prepared as above, but excluding exposure to the primary antibody step of the preparation. Sections were washed twice and then counterstained using 2 % uranyl acetate and lead citrate, before washing in ultra pure water, 6 grids were performed for each sample. Microscopy was done using a 1210 Joel transmission electron microscope. At least ten photos of each sample were taken.

6.3.4 Gene expression of FtsZ, Atl and Gcp using Reverse Transcriptase PCR one and four hours after exposure to treatment.

EMRSA-15 was grown overnight in TSB at 37°C. The culture was then diluted the following morning 2:50 in TSB and incubation continued until optical density reached 0.6
– 1.0 against a H$_2$O blank (Cecil spectrophotometer). Cultures of 50 ml in 500 ml conical flasks were grown in a Brunswick orbital incubator at 37°C at 200 rpm.

Once the required optical density was achieved, 1 ml of cells were spun down at 13000 g for two minutes (MSE Microfuge, Sanyo), supernatant was removed and treated using a SV Total RNA isolation system (Promega, Southampton), and the pellet resuspended in 60 µl of 10 mg/ml lysozyme (Sigma) plus 60 µl 10 mg/ml solution lysostaphin made up in super pure water (1 mg lysostaphin + 100 µl super pure water). This was incubated for 10 minutes at room temperature. To this 75 µl of RNA lysis buffer was added followed by 350 µl RNA dilution buffer. 200 µl 95% ethanol was added and mixed with pipette. The cleared lysate was then transferred to a spin column and spun at 13000 rpm for 2 minutes (MSE Microfuge, Sanyo). The spin basket was removed from the assembly and liquid from the collection tube was discarded, the spin basket replaced and 600 µl RNA wash solution added and spun 13000 rpm for 1 minute. Liquid from spin basket was again discarded. 50 µl DNAse incubation mix was applied to membrane of spin basket (5 µl DNase 1 + 40 µl Yellow core buffer + 5 µl 0.09 M magnesium chloride). Once on the membrane the tube was incubated for 25 minutes at room temperature. Then 200 µl DNase stop solution was added and spun 13000 rpm for one minute followed by 600 µl RNA wash solution spun 13000 rpm for one minute. The collection tube was then emptied, followed by addition of 250 µl RNA wash solution spun at 13000 rpm for 2 minutes. The cap was then cut from the tube and spin basket transferred to elution tube. 100 µl nuclease free water was added to membrane, spun 13000 rpm for 1 minute. Spin basket was then discarded. DNA carryover detected so additional step was added. To 100 µl RNA from SV RNA isolation kit (Promega, Southampton), 10 µl of 10x DNase buffer was added followed by 2 µl of turbo DNase (Ambion, Applied biosystems, Warrington) this was incubated for
30 mins at 37°C on the thriller. To this 1 µl of turbo DNase was added followed by 30 mins at 37°C on the thriller, 20 µl of inactivation reagent was added and incubated at room temperature for 2 minutes before spinning at 13000 g for 2 minutes, the supernatant was decanted into a clean tube and frozen at -70 °C.

Each sample was then treated using the Promega Access RT-PCR kit following the kit protocol. House keeping gene primers used were for shikimate dehydrogenase and acetyl CoA acetyltransferase, then experimentally designed primers (Table 10) for FtsZ, Atl and Gcp, were added to each tube.

Negative and positive controls were run from the kit. Test samples minus the reverse transcriptase were also run in Peltier thermal cycler to ensure the bands seen were produced by RNA not by residual DNA. Cycling programme was 45°C for 45 minutes, 94°C for 2 minutes then 94°C for 30 seconds, 53°C for 60 seconds, 68°C for 2 minutes repeated for 30 cycles then 68°C for 7 minutes. PCR products were run on a 3% agarose gel at 75 v for one hour and stained with ethidium bromide final concentration of 0.5 µg/ml and visualized using Quantity one software on a GelDoc 2000 (Bio-Rad, Hemel Hempstead). The bands were analysed by using a density trace which gave the intensity of each band (see figure 29). The peaks on the density trace gave a value of intensity that was then used to compare the intensity of the gene of interest to the intensity of the house keeping genes at 1 and 4 hours after treatment with either manuka honey artificial honey and compared to those readings taken from those cells that had no treatment (control cells). Results were tested for statistical significance using Microsoft Minitab, two way T-test.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Primer Sequence</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shikimate dehydrogenase</td>
<td>Forward</td>
<td>ATCGGAAATCCTATTTTCACATTC</td>
<td>513</td>
</tr>
<tr>
<td>Shikimate dehydrogenase</td>
<td>Reverse</td>
<td>GGTGTGTATTAATAACGATATC</td>
<td></td>
</tr>
<tr>
<td>Acetyl CoA acetyltransferase</td>
<td>Forward</td>
<td>CAGCATACAGGACACCTATTGGGC</td>
<td>516</td>
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<tr>
<td>Acetyl CoA acetyltransferase</td>
<td>Reverse</td>
<td>CGTTGAGGAATCGATACTGGAAC</td>
<td></td>
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<tr>
<td>FtsZ</td>
<td>Forward</td>
<td>ACTAGGAGAAATTTAAATG</td>
<td>490</td>
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<tr>
<td>FtsZ</td>
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<td>ACTGCAGCTTTTCATAGCTTC</td>
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<td>GCTGGTTATAGTTAGTTGATGATG</td>
<td>196</td>
</tr>
<tr>
<td>Atl</td>
<td>Reverse</td>
<td>GGTGTGTGCTGAAGCGCTAAAAG</td>
<td></td>
</tr>
<tr>
<td>Gcp</td>
<td>Forward</td>
<td>AGCAAATCGACCTTGCTGAT</td>
<td>135</td>
</tr>
<tr>
<td>Gcp</td>
<td>Reverse</td>
<td>CGAGATGACGCAGTAGGTTGA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 10: Primer sequences used to look at genes of interest in EMRSA-15 after treatment with and without honey:** Sequences used to amplify target RNA in treated and untreated cell cultures. First two sequences are house keeping genes used as a control during experiments (sequences adapted from Pinho and Errington, 2003; Enright et al, 2000; Zheng et al, 2007 using BRENDA).
6.4 Results

Non specific staining was not observed in any of the controls for fluorescence or gold labelling work.

6.4.1 Localisation of FtsZ by Immunofluorescence

The distribution of FtsZ in MRSA varied with time. Manuka-treated MRSA cells showed patterns of localization that were distinct from those observed in control cells (no treatment and artificial honey) (Figure 30). After 240 minutes the percentage of cells exhibiting the presence of FtsZ was significantly higher in manuka honey treated cells than in untreated cells using two way T test, \( p = 0.006 \). The position of FtsZ within treated cells was also
Figure 30: Fluorescent labelling of FtsZ within EMRSA-15 over a 24hr period after treatment with and with honey. Honey treated cells (open squares) show an initial increase in FITC labelling compared to control cells (open triangles) and artificial honey treated cells (open diamonds). However after twenty four hours the honey treated cells show a significant decrease in FITC labelling compared to the control cultures, graph shows +/- standard deviation.
distinct from untreated cell cultures and appeared consistently in a halo form around the cell suggesting extensive delocalized distribution (Figure 33) rather than the line and bar-like distribution seen in untreated cells. This was unlike results seen in those cells treated with artificial honey where there was no significant increase in percentage of cells stained compared to untreated cells was seen (p = 0.516). Conversely at 1440 minutes, the number of manuka honey treated cells expressing the FtsZ protein was clearly reduced to lower levels than the control cells, but those showing labelling were still in a delocalized halo form (Figure 30).

Images of cultures after 240 minutes incubation (Figures 31, 32 and 33) and after 1440 minutes (Figures 34, 35 and 36) are presented here to illustrate the observed changes in FtsZ localization.

In control cultures (untreated and incubated with artificial honey) (Figures 31, 34 and Figures 32, 36 respectively), FtsZ was present as infrequent partial and complete ring-like halo structures (Figure 31). Although the number of control cells increases between 240 minutes and 1440 minutes the distribution of FITC labelled FtsZ in these cells had not altered, it was still seen in bars and incomplete halos, therefore not delocalized (Figure 34). In MRSA treated with artificial honey for 1440 minutes the distribution of FtsZ was found to be in bars and some halos suggesting limited delocalization (Figure 33). Representative images are shown below to illustrate the effects observed during this experiment.
Figure 31: Localisation of FtsZ using immunofluorescence in EMRSA-15 at 240 minutes after treatment. a) Untreated MRSA-15 at 240 minutes. Blue = DAPI, Green = FITC (FtsZ).
Figure 32: Localisation of FtsZ using immunofluorescence in EMRSA-15 at 240 minutes after treatment. b) EMRSA-15 after 240 minutes at 37°C with TSB containing 10% (w/v) artificial honey.
Figure 33: Localisation of FtsZ using immunofluorescence in EMRSA-15 at 240 minutes after treatment. c) EMRSA-15 after 240 minutes in TSB containing 10% (w/v) manuka honey, note the presence of rings of delocalised FtsZ protein.
Figure 34: Localisation of FtsZ using immunofluorescence in EMRSA-15 at 1440 minutes after treatment. a) Untreated EMRSA-15 at 1440 minutes: note the presence of bars of FtsZ protein.
Figure 35: Localisation of FtsZ using immunofluorescence in EMRSA-15 at 1440 minutes after treatment. b) EMRSA-15 after 1440 minutes in TSB containing 10% (w/v) artificial honey.
Figure 36: Localisation of FtsZ using immunofluorescence in EMRSA-15 at 1440 minutes after treatment. c) EMRSA-15 after 1440 minutes in TSB containing 10 % (w/v) manuka honey.
6.4.2 Gold labelling Localisation of FtsZ using Transmission Electron Microscopy of Whole Cells

The electron micrographs showed that compared to untreated control MRSA cells (Fig. 39), the distribution and extent of FtsZ on the surface of whole cells was unaffected by treatment of those cells with artificial honey, so looked the same as control cells (p = < 0.05) (Fig. 40). However the gold labelling seen on manuka honey treated cells was significantly reduced using two way T test (p >0.001) (Fig. 41). The difference between the gold labelling in (control) artificial honey treated cells and manuka honey treated cells can be seen in Figure 37 (For gold counts see appendix 1).

6.4.3 Gold Labelling Localisation of FtsZ using Transmission Electron Microscopy of Thin Sections

Examining the images of the thin sections (Fig.44) showed treatment of cells with manuka honey did not seem to affect either the extent or distribution of FtsZ in MRSA compared to control cells (p = < 0.05) (Fig. 42). However but less enzyme was present in cells treated with artificial honey compared to control cells using two way T test (p >0.001) (Fig. 43). The difference between the gold labelling in (control) artificial honey treated cells and manuka honey treated cells can be seen in Figure 38 (For gold counts see appendix 2).
Figure 37: Gold labelling Localisation of FtsZ using Transmission Electron Microscopy of S. aureus Whole Cells after 1440 minutes treatment. Average number of gold particles per cell on whole cell TEM labelling FtsZ. ** No statistically significant difference in numbers of gold particles seen on control and artificial honey treated cells (p = < 0.05). * Significantly different numbers of gold particles were seen on manuka honey treated cells, 60% lower (p =>0.001).
Figure 38: Gold Labelling Localisation of FtsZ using Transmission Electron Microscopy of Thin Sections 1440 minutes after treatment. Average number of gold particles per TEM labelling FtsZ. *No statistically significant difference in numbers of gold particles seen on control and manuka honey treated cells (p = < 0.05). **Significantly different numbers of gold particles were seen on artificial honey treated cells, 50% lower (p= >0.001).
Figure 39: Surface gold labelling of FtsZ in EMRSA-15. Surface localization of FtsZ in whole cells of MRSA that were incubated with TSB for 1440 minutes.
Figure 40: Surface gold labeling of FtsZ in EMRSA-15. Cells incubated with TSB containing 10% (w/v) artificial honey for 1440 minutes.
Figure 41: Surface gold labeling of FtsZ in EMRSA-15. Cells incubated with TSB containing 10% (w/v) manuka honey for 1440 minutes. All images were captured at 20,000 times magnification.
**Figure 42: Intracellular gold labelling of FtsZ in EMRSA-15.** Intracellular localization of FtsZ in thin sections of MRSA that were incubated with TSB for 1440 minutes. All images were captured at 32,000 times magnification.
Figure 43: Intracellular gold labelling of FtsZ in EMRSA-15. Intracellular localization of FtsZ in thin sections of MRSA that were incubated with TSB containing 10% (w/v) artificial honey for 1440 minutes. All images were captured at 32,000 times magnification.
Figure 44: Intracellular gold labelling of FtsZ in EMRSA-15. Intracellular localization of FtsZ in thin sections of MRSA that were incubated with TSB containing 10% (w/v) manuka honey for 1440 minutes. All images were captured at 32,000 times magnification.
6.4.4 Gene expression of FtsZ using Reverse Transcriptase PCR one and four hours after exposure to treatment.

Gels were analysed using Gel Doc, Quantity one software (Biorad, Hertfordshire, UK).

Bands were selected as regions of interest and comparative intensities were recorded.

The FtsZ primers showed that the honey treated cells showed roughly a 10% increase in FtsZ expression compared to control and artificial honey treated cells, gels done at least in duplicate to ensure reproducibility of results (Figure 46-47). This increase was seen at both 1 and 4 hours after treatment, however this difference was not statistically significant p = >0.005. Bands from top of the gel to bottom are Acetyl CoActeyl Transferase, Shikimate dehydrogenase and FtsZ (Figure 45).

Lane 1 – Colour burst electrophoresis size markers (Sigma)

Lane 2 - Negative control

Lane 3 - Positive control (mecA 250bp)

Lane 4 - mRNA from control treated cells for Acetyl CoActeyl Transferase (516bp), Shikimate dehydrogenase (513bp) and FtsZ (490bp).

Lane 5 - mRNA from artificial honey treated cells for Acetyl CoActeyl Transferase (516bp), Shikimate dehydrogenase (513bp) and FtsZ (490bp).

Lane 6 - mRNA from manuka honey treated cells for Acetyl CoActeyl Transferase (516bp), Shikimate dehydrogenase (513bp) and FtsZ (490bp).

**Figure 45: An example PCR gel of FtsZ.** Example of PCR gel showing gene expression of FtsZ in EMRSA-15, 1 hour after treatment.
Figure 46: mRNA expression of FtsZ 60 minutes after treatment. Percentage expression of FtsZ mRNA compared to expression of house keeping gene Acetyl co enzyme A, after one hours treatment with manuka honey, artificial honey or no treatment (control). No significant difference in expression between any of the treatments (p = < 0.05).

Figure 47: mRNA expression of FtsZ 240 minutes after treatment. Percentage expression of FtsZ mRNA compared to expression of house keeping gene Acetyl co enzyme A, after four hours treatment with manuka honey, artificial honey or no treatment (control). No significant difference in expression between any of the treatments (p = < 0.05).
6.4.5 Localisation of Endo-B-N-Acetylg glucosaminidase by Immunofluorescence

The immunofluorescence showed that there was little change in the amount of labelling seen in control cells and that those treated with 10% (w/v) artificial honey in TSB over time, nearly a 100% of cells showed labelling at both 240 minutes and 1440 minutes (Figures 48 & 51). The artificial honey treated cells also maintained a high level of labelling over time similar to the control cells (Figures 49 & 52). There was no significant difference in the labelling of honey treated cells compared to control cells at 240 minutes p = > 0.05) (Figure 50). After 240 minutes the percentage of cells exhibiting the presence of Endo-B-N-Acetylg glucosaminidase were not significantly lower in manuka honey treated cells than in untreated cells using two way T test, (p = 0.69). However a decrease in the amount of labelling was seen in honey treated cells over time, at 1440 minutes there were virtually no cells showing labelling, showing significant difference using a two way T test p = <0.0001 (Figure 53). Figure 54 shows the decrease in fluorescence seen in honey treated cells over time compared to both control and artificial honey treated cells.

Representative images are shown below to illustrate the effects observed during this experiment.
Figure 48: Localisation of Endo-B-N-Acetylglucosaminidase using immunofluorescence in EMRSA-15 at 240 minutes after treatment. a) Untreated MRSA-15 at 240 minutes. Blue = DAPI, Green = FITC (Endo-B-N-Acetylglucosaminidase).
Figure 49: Localisation of Endo-B-N-Acetylglucosaminidase using immunofluorescence in EMRSA-15 at 240 minutes after treatment. b) EMRSA-15 after 240 minutes at 37°C with TSB containing 10% (w/v) artificial honey.
Figure 50: Localisation of Endo-B-N-Acetylglucosaminidase using immunofluorescence in EMRSA-15 at 240 minutes after treatment. c) EMRSA-15 after 240 minutes in TSB containing 10% (w/v) manuka honey.
Figure 51: Localisation of Endo-B-N-Acetylglucosaminidase using immunofluorescence in EMRSA-15 at 1440 minutes after treatment. a) Untreated MRSA-15 at 1440 minutes.
Figure 52: Localisation of Endo-B-N-Acetylglucosaminidase using immunofluorescence in EMRSA-15 at 1440 minutes after treatment. b) EMRSA-15 after 1440 minutes at 37°C with TSB containing 10% (w/v) artificial honey.
Figure 53: Localisation of Endo-B-N-Acetylglucosaminidase using immunofluorescence in EMRSA-15 at 1440 minutes after treatment. c) EMRSA-15 after 1440 minutes in TSB containing 10% (w/v) manuka honey.
Figure 54: Fluorescent labelling of B-N-endoacetylglucosaminidase within EMRSA-15 over 24 hr period after treatment with and without honey. Cells treated with honey (open circles) show a decreasing level of FITC labeling over the twenty four period of treatment and observation when compared to the control cells (open squares) and the artificial honey treated cells (open diamonds). Error bars indicate standard deviation.
6.4.6 Gold labelling Localisation of Endo-B-N-Acetylglucosaminidase using Transmission Electron Microscopy of Whole Cells

Although there were no statistically significant differences in the extent of labelling for endo-beta-N-acetylglucosaminidase between untreated and honey treated cells (Figure 55), a dispersed distribution of gold was seen on the images of control and artificial honey treated cells (Fig. 57 and 58). There was difference in distribution in manuka honey treated cells where the enzyme was confined to cell junction regions (Fig. 59). Numbers of gold particles per cell were counted and compared using two way T test in Microsoft Minitab version 14, p = > 0.05 (For gold counts see appendix 3).

6.4.7 Gold Labelling Localisation of Endo-B-N-Acetylglucosaminidase using Transmission Electron Microscopy of Thin Sections

Differences were seen in the levels of endo-beta-N-acetylglucosaminidase in thin sections (Figure 56). Compared to untreated cells (Fig. 60), less enzyme was seen in cells treated with artificial honey (p >0.024) (Fig.61) but higher levels were found in cells treated with manuka honey (p = 0.003) (Fig. 62) (For gold counts see appendix 4).
Figure 55: Gold labelling Localisation of Endo-B-N-Acetylglucosaminidase using Transmission Electron Microscopy of Whole Cells

Average number of gold particles per cell on whole cell TEM labelling Endo-B-N-Acetylglucosaminidase. 

* No statistical significance was seen in the numbers of particles of gold between control cells and manuka honey treated cells $p = > 0.05$.

** No statistical significance was seen in the numbers of particles of gold between control cells and artificial honey treated cells $p = > 0.05$. 
Figure 56: Gold Labelling Localisation of Endo-B-N-Acetylglucosaminidase using Transmission Electron Microscopy of Thin Sections. Average number of gold particles per cell on thin sections cell TEM labelling Endo-B-N-Acetylglucosaminidase. **Statistically different numbers of gold particles seen on artificial honey treated cells than on control cells 50% higher (p >0.024). * Statistically different levels were found in cells treated with manuka honey 80% higher (p = 0.003).
Figure 57: Surface gold labelling of Endo-B-N-acetylglucosaminidase in EMRSA-15. Surface localisation of Endo-beta-N-acetylglucosaminidase on whole cell EMRSA-15 that was incubated on TSB for 1440 minutes. All images at 20,000 times magnification.
Figure 58: Surface gold labelling of Endo-B-N-acetylglucosaminidase in EMRSA-15. Surface localisation of Endo-beta-N-acetylglucosaminidase on whole cell EMRSA-15 incubated in TSB containing 10% (w/v) artificial honey for 1440 minutes. All images were taken at 20,000 times magnification.
Figure 59: Surface localization of Endo-B-N-acetylglucosaminidase in EMRSA-15. Surface localization of Endo-B-N-acetylglucosaminidase on whole cells of EMRSA-15 that were incubated with TSB containing 10% (w/v) manuka honey. All images were captured at 20,000 times magnification.
Figure 60: Intracellular localisation of Endo-B-N-acetylglucosaminidase in EMRSA-15. Intracellular localization of Endo-B-N-endoaecetylglucosaminidase in thin sections of MRSA that were incubated in TSB for 1440 minutes. All images were captured at 32,000 times magnification.
Figure 61: Intracellular localisation of Endo-B-N-acetylglucosaminidase in EMRSA-15. Intracellular localization of Endo-B-N-acetylglucosaminidase in thin sections of MRSA that were incubated in TSB containing 10% w/v artificial honey for 1440 minutes. All images were captured at 32,000 times magnification.
Figure 62: Intracellular localisation of Endo-B-N-acetylglucosaminidase in EMRSA-15. Intracellular localization of Endo-B-N-acetylglucosaminidase in thin sections of MRSA that were incubated in TSB containing 10% w/v manuka honey for 1440 minutes. All images were captured at 32,000 times magnification.
6.4.8 Gene expression of *Atl* and Gcp using Reverse Transcriptase PCR one and four hours after exposure to treatment.

Gels were analysed using Gel Doc, Quantity one software (Biorad, Hertfordshire, UK). Bands were selected as regions of interest and comparative intensities were recorded (Figure 63 and 66). The *atl* primers used on honey treated and artificial honey treated cell extracts (Figures 64 and 65) did not show any significant difference in expression compared to control cell extracts. The gcp primers used on honey treated and artificial honey treated cell extracts (Figures 67 and 68) also did not show any significant difference in expression compared to control cell extracts. No statistically significant differences were seen between any of the treated cell when compared to control cells \( p > 0.05 \). This was the case after both 1 and 4 hours of treatment.

![Image of PCR gel showing gene expression of *atl*](image)

**Figure 63: PCR gel of *atl*. Example of PCR gel showing gene expression of *atl* in EMRSA-15, 1 hour after treatment.** Lane 1 – Negative control, Lane 2 - Acetyl co enzyme transferase (516bp) expression from control cells, Lane 3 - *atl* (196bp) expression from control cells, Lane 4 - Acetyl co enzyme transferase (516bp) expression from artificial honey treated cells, Lane 5 - *atl* (196bp) expression from artificial honey treated cells, Lane 6 - Acetyl co enzyme transferase (516bp) expression from manuka honey treated cells, Lane 7 - *atl* (196bp) expression from manuka honey treated cells, Lane 8 - Colour burst electrophoresis size markers (Sigma).
Figure 64. mRNA expression of Atl 60 minutes after treatment. Percentage expression of Atl mRNA compared to expression of house keeping gene Acetyl co enzyme A, after one hours treatment with manuka honey, artificial honey or no treatment (control). No significant difference in expression between treatments (p = > 0.05).

Figure 65. mRNA expression of Atl 240 minutes after treatment. Percentage expression of Atl mRNA compared to expression of house keeping gene Acetyl co enzyme A, after four hours treatment with manuka honey, artificial honey or no treatment (control). No significant difference in expression between treatments (p = > 0.05).
Figure 66: Example of PCR gel of Gcp products extracted from EMRSA-15, 1 hour after treatments. PCR gel showing gene expression of gcp relative to housekeeping gene in EMRSA-15, tested at 1 hour after treatment with and without honey.
Figure 67. mRNA expression of gcp in EMRSA-15, 60 minutes after treatment.
Percentage expression of gcp mRNA compared to expression of house keeping gene Acetyl coenzyme A, after one hour’s treatment with manuka honey, artificial honey or no treatment (control). No significant difference in expression between treatments (p > 0.05).

Figure 68. mRNA expression of gcp in EMRSA-15, 240 minutes after treatment.
Percentage expression of gcp mRNA compared to expression of house keeping gene Acetyl coenzyme A, after four hours treatment with manuka honey, artificial honey or no treatment (control). No significant difference in expression between treatments (p > 0.05).
6.5 Discussion

FtsZ is an essential cell division protein required for normal bacterial division and cell viability. It has been shown that disruption of FtsZ by several substances and most recently curcumin (a naturally occurring polyphenolic compound) has led to an inhibition of cell division and proliferation by interruption of the Z ring (Rai et al, 2008). Cell division therefore is a valid target for antimicrobial therapy. If honey should inhibit cell division or Z ring formation it could lead to death of the cell or reduction in proliferation of the cells, thus providing host defences with an opportunity to act effectively to remove invading bacterial cells.

The images taken in this study have shown that treatment of cells with manuka honey causes a clear difference in FtsZ expression when compared to control cultures (p = <0.05). The fluorescence microscopy indicates that 240 minutes after exposure to inhibitory concentration of manuka honey that the localisation of FtsZ has markedly changed to a halo form (which suggests delocalisation of the protein) in contrast to the control culture where the protein was localised as bars and semi-halos.

However although there is show a clear difference in distribution between control cells and manuka honey treated cells, the differences in the amount of FtsZ produced shown by the fluorescence microscopy and the TEM can lead to two interpretations.

The first scenario is that fluorescence microscopy demonstrates internal labelling of FtsZ and so as FtsZ is an internal protein. This is true then the drop in FtsZ seen at 1440 minutes
directly contradicts what is seen in the gold labelled thin sections at 1440 minutes as no significant difference in amount of FtsZ labelling is seen using this method.

The alternative scenario is that the FITC is in fact labelling external FtsZ on the surface of the cells, if this was the case then both the fluorescence microscopy and TEM of whole cells show that at 1440 minutes a significant reduction in FtsZ is seen in manuka honey treated cells.

To investigate whether the labelling is indeed external to the cell, immunofluorescence could be repeated but without using the lysostaphin step to see if pictures taken retain the same appearance as those treated with lysostaphin. Similar appearance would indicate that the labelling was external, on the surface of the cells. Alternatively GTP/FtsZ fusion mutants could be used to monitor the expression and movement of FtsZ in the cell to give a better picture of what occurs before and after treatment with manuka honey. Without these further experiments it can still be concluded that the treatment with manuka honey does have a significant effect after 1440 minutes. Also that honey contains one or more components that affect FtsZ, distinct from the sugars found in honey as sugars alone gave pictures that showed a profile different to the honey treated pictures.

The increase in FtsZ expression seen in the PCR although small supports data seen in early studies. It would appear that after treatment with manuka honey *S. aureus* cell transiently increased production of FtsZ above normal levels.

It is possible that the decrease and delocalized distribution of FtsZ by 1440 minutes contributes to the cells inability to complete cell division. Previously FtsZ polymerization
was shown to be blocked by viriditoxin, leading to cell death (Wang et al, 2003). The different pattern of FtsZ localization detected in manuka-treated MRSA, suggests that manuka honey contains one or more as yet unknown components that do influence FtsZ localization or polymerization.

These electron micrographs showed that although treatment with honey does affect the localisation of FtsZ on the surface of the cells, (Fig. 41) compared to the control (Fig. 39 and 40) it does not affect the formation of septa within the cell. This may indicate that the failure to separate could be due to an inability of the FtsZ to recruit later division proteins such as FtsI or FtsN (Harry et al, 2006). Alternatively if the septal recruitment pathway is complete then the hydrolysis and change in conformation conferred on FtsZ by GTP may not be occurring. This would lead to the membrane not undergoing constriction and invagination and be seen as cells that have septa but have not divided. This was observed in these experiments.

The distribution of FtsZ inside the manuka honey treated cells (Fig. 44) is comparable with that of the control cells (Fig. 42 and 43) and supports the hypothesis that the septa are forming normally and the interruption in the cell cycle comes afterwards at the point of cell division. The lack of FtsZ seen outside of the cell in the honey treated cells could be an indication that although the Z ring has formed correctly there is an effect on the disassembly of the Z ring and perhaps a failure of the ring to constrict and separate therefore not leaving any FtsZ free to be pumped out of the cell as the cell divides and the Z ring disassembles, as seen in the control cells.
Others have shown that FtsZ is inhibited using GTP analogues, reduced bundling of protofilaments and alterations of secondary structure, also it has been studied in depth using mutant cells (Blaauwen, *et al.*, 1999; Margolin, 2003). The affected bacteria in these cases either die or often form long filaments of bacteria rather than their usual form. This extension of cell length was not seen in the above experiments and therefore the honey is probably acting on a different target. The target could be on one of the other Fts proteins recruited to the Z ring but the effect might not be observed as some conformational changes and point mutations within the proteins can compensate for some loss of integrity within the division process (Bernard *et al.*, 2007).

It is also noticeable in the electron micrographs that the manuka honey treated cells have an enlarged appearance. This indicates that although the cells are failing to divide in the normal manner there is at least some growth continuing to occur after the point where the cells should have split into two.

It is likely in this case that the increase in cells with complete septa (*p* = seen earlier in the TEM experiments was due to a change in cell cycle after the formation of the Z ring. Potentially the action of the murein hydrolases that digest the peptidoglycan bonds needed to separate the daughter cells at cytokinesis had been attenuated by some as yet unknown mechanism.

The images shown here indicate that production of endo-B-N-acetylglucosaminidase within EMRSA-15 was clearly affected by treatment with manuka honey. This effect is also clearly not caused solely by the sugars found in honey, as the pictures seen from the artificially treated cells were distinct from the images of honey treated cells.
The fluorescence indicates that the endo-beta-N-acetylglucosaminidase within manuka honey treated cells is significantly less than controls by 1440 minutes exposure.

Again once the results (Figure 48 -53) are compared to those seen in the gold labelling it is apparent that the results are contradictory, as the fluorescent labelling is seen within the cells. The thin sections indicate a significant increase in enzyme compared to control cells.

However, if it is assumed that the fluorescent labelling had not entered the cells then the results as the results for FtsZ show a more similar outcome. Although no statistical significant reduction in labelling on the surface of the gold labelled cells (p = < 0.05) was found, there is a qualitative difference in the amount of gold (ie in the distribution of the enzyme), which might indicate a change in the targeting of the enzyme to its substrate that correlates with the fluorescence results. It is also apparent that the de-localisation of fluorescence seen in chapter 5 did not occur because the fluorescence was present as bars and lines within cells and not in halos around the cells. This suggests that manuka honey exerted a different effect on endo-beta-N-acetylglucosaminidase compared to on FtsZ.

These results are unexpected and to clarify what is being seen by fluorescence and gold labelling further experiments are needed. For example microarray analysis might give an insight into the change in expression of this enzyme and others and real time PCR might help to show how early and how strongly the enzyme was expressed within the cell cycle, although do not help identify the locaion of the enzymes within the cell.

Upon simple inspection of the gold labelling patterns it is curious that even though endo-beta-N-acetylglucosaminidase levels are markedly elevated within manuka treated MRSA
cells (Fig. 2b H), these cells fail to complete cell separation. This could be due to an alteration in the enzyme or enzyme substrate (meaning the enzyme would be unable to recognise the substrate), leading to a failure of the enzyme to bind to its target site (Zheng et al, 2007) and hints that control of production of endo-beta-N-acetylglucosaminidase could have been altered.

It is possible that the targeting of this enzyme to its substrate was altered by honey treatment. The gold labelling shows an increased amount of gold in the intracellular areas of the cell (thin sections) and a reduced amount of extra cellular enzyme in the whole cell pictures showing the surface associated enzyme. This was not expected or seen in control cells as endo-beta-N-acetylglucosaminidase is a cell surface associated and secreted enzyme when in its mature form (Vollmer et al, 2008). Therefore the change seen in honey treated cells could imply an alteration in the localisation of endo-beta-N-acetylglucosaminidase, or a failure of the cell to transport it to the site of action.

There is also potential for aberrations earlier in the cell cycle to have impacted on this enzyme. For instance the absence of FtsZ on the surface of manuka honey treated cells shown earlier in the chapter has ramifications for the sequence of events leading to recruitment of endo-beta-N-acetylglucosaminidase, possibly leading to failure of this enzyme to localise correctly. This has been seen before in previous studies with tetracycline, showing that while tetracycline produces an increase in atl transcription it actually causes a decrease in autolysis possibly due to inhibition of atl localisation at the septal region (Yamada et al, 2001).
It has been known for a long time that perhaps up to 90% of the autolytic activity found in *S. aureus* is not essential for growth of the cell (Chatterjee *et al.*, 1976). The results seen here seem to support that theory and indicate that the role of endo-beta-N-acetylglucosaminidase occurs at a late stage of cell division after the separation of the genome and the formation of the septa. From the TEMs presented in chapter 4 it is apparent that manuka honey treated cells still formed septa, despite a drop in the levels of cell surface enzyme. Also it is known that like lytic transglycosylases one or more of the bacterial hydrolases can be removed without eliciting an immediately lethal effect (Scheurwater *et al.*, 2007).

These results could denote an effect on the translational control of endo-beta-N-acetylglucosaminidase and the *atl* operon, it may suggest that there is a generalised down regulation of production of *atl* which would lead to the reduced enzyme seen in both fluorescence and surface gold labelling.

Alternatively observations of change in gene expression could be due to an alteration in something like Gcp which may be involved in the post translational control of endo-beta-N-acetylglucosaminidase (Zheng *et al.*, 2007). If this were the case then the honey treated cells may well be producing as much *atl* as control cells but proteolytic cleavage and targeting of the two enzymes created from this may not be correct. The reduced endo-beta-N-acetylglucosaminidase seen in the results could indicate that manuka honey induces a generalised stress response from the cell.

Studies have suggested that the *atl* is a member of the cell wall stress stimulon and in times of stress *atl* production is reduced to conserve the cells peptidoglycan in the presence of
chemicals that might cause lysis. It is known that decreased autolytic activity is thought to reduce susceptibility of *S. aureus* to antibiotics; transcription of *atl* is lower in vancomycin intermediate *S. aureus* compared to the sensitive strain (Ledala *et al*, 2006). It was evident throughout this study that manuka honey treated cells had an enlarged appearance, larger than control cultures. This also indicates that although the role of the enzyme has been inhibited and cell separation does not occur the cells still continue to grow for some time after the failure to divide at the usual point.

As endo-beta-N-acetylglucosaminidase is formed via proteolytic processing of Atl. It is possible that it fails to go through the intermediate stage of processing forming a 115 kDa and a 85 kDa protein. Present an inactive form unable to digest peptidoglycan bonds but still being recognised by the antibodies used.
7. An Investigation into the Effects of Manuka Honey and Methylglyoxal on protein Expression in Meticillin resistant *Staphylococcus aureus*

7.1 Introduction

The prevalence of strains of *Staphylococcus aureus* with resistance determinants to multiple antibiotics both in health care settings and in the community represents a serious health care threat. Because of this problem, it is important that there is a detailed understanding of the physiology of these bacteria. The post genomic era for *S. aureus* started in 2001 with the publication of the first 2 genome sequences. Over the years numerous genomes of pathogenic bacteria have been made available and there are at least 10 complete *S. aureus* genomes now available (Kuroda *et al*, 2001; Baba *et al*, 2002; Holden *et al*, 2004; Gill *et al*, 2005; Diep *et al*, 2006). The genome encodes around 2600 proteins with up to 40% of these having a known function and around a third appear to be unique to *S. aureus* (Heckler *et al*, 2003). This kind of information provides a basis for the understanding of bacterial metabolism, adaptability, mechanisms of resistance and pathogenicity.

The investigation of the proteins expressed in *S. aureus* during its life cycle and while under pressure from antimicrobials could potentially help to elucidate mechanisms of virulence and also mode of action of those antimicrobials, by observing which proteins are up or down reugulated in response to treatment.
Two dimensional electrophoresis is a well established technique for the separation and quantification of proteins and allows comparative analysis of the proteomics of the cell (Gatlin et al, 2006). It has previously been shown to be a successful technique for investigating the effects of novel antimicrobials against *S. aureus*, also in comparative proteome mapping of differing *S. aureus* strains when grown in the presence and absence of environmental challenges (Cho et al, 2008; Cordwell et al, 2002) indicating the up or down regulation of specific proteins.

In this chapter a 2-D electrophoresis to investigate the effect of manuka honey on EMRSA-15. However early in 2009 two papers were published that identified methylglyoxal (MGO) as a previously unknown antibacterial factor in manuka honey (Mavric et al 2008; Adams et al 2008). As the work described in the previous chapters was performed without this knowledge, it was decided to extend my this study for 6 months to provide an opportunity to investigate whether any of the observed effects were attributable to MGO. In the time available it was decided to repeat the time-kill curve and the transmission electron microscopy using artificial honey containing the predicted concentration of MGO for the manuka honey that had been used throughout this thesis. The effect of MGO on protein expression in MRSA was tested concurrently with manuka honey and artificial honey.

### 7.2 Aims

The aims were to observe the differences in protein expression between untreated, manuka honey treated, MGO treated or artificially honey treated EMRSA-15 four hours after treatment, using 2-D Gel electrophoresis. Also to determine whether any protein changes detected by 2-D electrophoresis might explain any of the structural changes seen in electron
micrographs. Before this was attempted, it was necessary to ascertain whether MGO affected MRSA in the same manner as manuka honey either kinetically or structurally.

7.3 Methods

7.3.1 Time Kill Curves

From an overnight culture of EMRSA-15 (Oxoid, Cambridge, UK) a 40 µl sample was inoculated into each of three conical flasks containing either 20 ml nutrient broth alone, nutrient broth containing 10% (w/v) manuka honey or nutrient broth containing 10% (w/v) artificial honey and 614 mg/kg MGO. To work out the correct amount of MGO added to the artificial honey to get the equivalent of the amount found in the manuka honey that we were using, a calculation was performed. This involved using a graph published by Adams et al, 2007. This graph gave the mg/kg MGO in relation to the UMF factor. The UMF of our manuka honey was 18 which gave a reading of 350 mg/kg. However the published graph was based on work that had two errors in it, due to an arithmetical error in the spreadsheet used for the MGO assays all the results were too low, this required that the figure given was multiplied 1.5 to get the correct results. It was also discovered that the solution of MGO purchased from Sigma being used to determine the mk/kg labelled as 40% was 40% by weight and not 40% by volume as would be expected, this meant that the final answer had to have 17% of the figure added to it to correct that, the calculation to find the mk/kg of MGO in our sample is seen below:

Honey with UMF factor of 18 contained 350 mg/kg MGO

\[
350 \times 1.5 = 525 \\
(525/100) \times 17 = 89.25 \\
525 + 89.25 = 614.25 \text{ mg/kg}
\]
After this correction the amount of MGO needed in artificial honey was 614 mg/kg, which was the same as 6.14 mg per 10 g artificial honey.

Samples were removed at known time intervals and total viable counts were obtained using the Miles and Misra surface drop method (Drabu & Blakemore 1990; Levett 1991; Tweats, Green & Muriel 1981). Time-kill curves were plotted.

### 7.3.2 Collection of Cells for Electron Microscopy
Specimens were collected at 7 time points from Time 0 to 24 hours from cultures of EMRSA-15 in TSB containing 10% (w/v) artificial honey and MGO. The methods used here were identical to those used in chapter 4 and the data was incorporated with that derived previously and plotted. The results were then analysed for statistical significance using a one way ANOVA in Microsoft Minitab version 14.

### 7.3.3 Collection of Cells for 2-D Gel Electrophoresis
The methods used for the two dimensional gel electrophoresis were adapted from Kohler et al, 2003). A single EMRSA-15 colony from a TSB plate was inoculated into 10 ml sterile TSB overnight. Then 2 ml overnight culture was suspended in 50 ml total volume of TSB in a 500 ml flask in an orbital incubator at 37°C, at 100 rpm for 24 hours. This culture was then increased to 100 ml TSB by adding 50 ml sterile TSB at 37°C for control culture, or 50 ml sterile TSB at 37°C containing 10 g manuka honey to give a final concentration of 10% (w/v) honey for the honey test cells. These were then incubated in the orbital incubator under the same condition for a further four hours.
The optical density was recorded against TSB as a blank at 550 nm. The cells were harvested by centrifugation at room temperature in a Sorvall RC 5B centrifuge for 5 minutes at 10,000 g. The resulting pellet was re-suspended in 10 ml sterile water. Cells were then treated in a bead beater (Biospec, Bartlesville, USA) according to operating instructions. Briefly the desired amount of 0.1 ml glass beads were weighed into the mini bead beater tube, the harvested suspension of EMRSA-15 cells was added to fill the tube, avoiding any air pockets and this was then placed in bead beater and treated at maximum speed for 3 x 1 minute with one minute cooling in ice after each of the three treatments.

Previous experiments had determined that 0.1mm glass beads gave a higher protein yield than the other types and diameters of bead available for the bead beater. Tubes were then spun in a microfuge (MSE Microfuge, Sanyo) at 13,000 g for 4 minutes, multiple supernatants were pooled and spun at 50000 x g for 2 hours at 4°C in the Sorvall RC 5B.

Protein concentration of extracts was determined using the Bradford method using Bio Rad kit and a microtitre plate technique as before (Chapter 3).

### 7.3.4 First Dimension Gel Electrophoresis

The appropriate amount of sample was added to re-hydration buffer to give a final volume of 200 µl, containing 150 µg of protein. This was roughly 25 µl of sample to 175 µl buffer (For buffer see table 11).
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
<th>Concentration/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Re-hydration buffer</strong></td>
<td>Urea 8 M</td>
<td>4.8 g</td>
</tr>
<tr>
<td></td>
<td>CHAPS 2% w/v</td>
<td>0.2 g</td>
</tr>
<tr>
<td></td>
<td>DDT 50 mM</td>
<td>0.08 g</td>
</tr>
<tr>
<td></td>
<td>Biolyte ampholytes (Bio-rad) pH3-10, 0.2% w/v</td>
<td>0.05 ml</td>
</tr>
<tr>
<td><strong>Equilibrium buffer 1</strong></td>
<td>Urea 6 M</td>
<td>9.0 g</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl, pH 8.8, 0.375 M</td>
<td>1.48 g</td>
</tr>
<tr>
<td></td>
<td>SDS 2%</td>
<td>0.5 g</td>
</tr>
<tr>
<td></td>
<td>Glycerol 20%</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>DTT 2% w/v</td>
<td>0.5 g</td>
</tr>
<tr>
<td><strong>Equilibrium buffer 2</strong></td>
<td>Urea 6 M</td>
<td>9 g</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl, pH 8.8, 0.375</td>
<td>1.48 g</td>
</tr>
<tr>
<td></td>
<td>SDS 2%</td>
<td>0.5 g</td>
</tr>
<tr>
<td></td>
<td>Glycerol 20%</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>Iodoacetamide 2.5% w/v</td>
<td>0.63 g</td>
</tr>
<tr>
<td><strong>MOPS running buffer</strong></td>
<td>10x concentrated, pH 7.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MOPS</td>
<td>104.5 g</td>
</tr>
<tr>
<td></td>
<td>Tris base</td>
<td>60.6 g</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>3 g</td>
</tr>
</tbody>
</table>

*Table 11:* Buffers used in 2-D Electrophoresis protocol
The sample was pipetted into an 11 cm re-hydration tray, leaving 1 cm free at each end. An 11 cm pH 3-10 IPG strip (Bio-Rad) was laid gel side down onto the sample, the lid placed on top of the tray which was then incubated at room temperature for one hour. Once the sample was absorbed into the strip it was covered with mineral oil, the lid was replaced and the tray incubated at room temperature overnight.

Paper wicks were placed into a clean focusing tray over the electrodes and 10 µl of 18 mega ohm water was pipetted onto each wick. The IPG strip was then transferred into this clean focussing tray gel side down onto the wicks and covered with fresh 2 ml mineral oil. The whole tray was then placed into a focusing cell (Bio Rad Protean IEF Cell) and treated at 50 micro amps for 35,000 volt hours at 20°C.

7.3.5 Second Dimension Gel Electrophoresis

The IPG strips were removed from the focusing tray and blotted gently on wet blotting paper. The strip was then placed in the rehydration tray for 30 minutes gel side up in 3 ml equilibrium buffer 1 and gently shaken in an orbital incubator at room temperature. Equilibrium buffer one was removed from the tray and 3 ml of equilibrium buffer two was added to the strip and incubated at room temperature; again it was shaken gently by the orbital incubator for 30 minutes. The strips were then removed from the buffer and dipped into a tube containing MOPS running buffer before being transferred into a precast criterion gel cassette (Criterion XT Precast cell, 4-12% Bis – Tris, Bio Rad) and sealed with molten agarose. The strips were covered in running buffer and left for 10 minutes to set. Running
buffer was added to the tank and the gels were then run at 200 v, for 55 minutes, in a Bio-Rad Criterion cell.

Once removed from the gel tank, the gels were stained using simply safe blue stain (Invitrogen, Paisley, UK) in the following way:

The gel was covered in 100 ml ultrapure water and microwaved on high (950-1100 watts) for one minute. It shaken on the orbital mixer for one minute, and the wash was discarded. This step was repeated twice more. To the washed gel, 20-30 ml simply blue safe stain (Invitrogen, Paisley, UK) was added and the gel microwaved for 1 minute. The gel was then incubated on the orbital mixer for 10 minutes, and then washed in 100 ml ultra pure water for 10 minutes. To this 100 ml ultra pure water 20 ml 20% (w/v) NaCl was added and incubated for 10 minutes. To obtain a clearer background the gels were washed again in ultra pure water for one hour. The gel was then visualised using the UVP AutoChemi, gel doc system and analysed using PDQuest Basic 8.0 software. This software created replicate groups of gels and then detected the numbers of spots present in each gel, and their intensity compared to a selected master (control gel). This allowed identification of those spots up or down regulated using gels of untreated cells as the control. The possible identity of some spots was speculated upon based on literature searches, overlaying already published profiles with identified spots onto the profiles generated here. However without further analytical techniques definitive identifications of spots were not possible.

7.4 Results

7.4.1 Time Kill Curves

For the time to kill study incubation in 10% w/v manuka honey and nutrient broth alone were used as controls to compare to the MGO. (Figure 69). Although MGO did inhibit EMRSA-15, the extent of inhibition seemed to be less than that of manuka honey.
Figure 69: Time kill curve of EMRSA-15 with and without artificial honey containing MGO: Viable cell counts of EMRSA-15 incubated in nutrient broth (NB) (filled diamonds), NB containing 10 % (w/v) manuka honey (filled squares), NB containing 10 %(w/v) artificial +MGO honey (filled triangles).
7.4.2 Transmission Electron Microscopy

For each time point 6 sections were cut and a minimum of 9 TEM micrographs were taken for each sample at every time point. The percentage of cells with septal components seen in all samples at time 0 varied between 35 – 50%. In the untreated cells the percentage with septal components remained between 35 -50% Those cells treated with 10% (v/v) artificial honey plus MGO did not show any significant difference in septa formation from control cells (Figure 70). Compared to the untreated control cells the numbers of septal components in cells treated with 10% (w/v) artificial honey containing MGO were not significantly different with p = 0.625. However those cells treated with 10% (w/v) artificial honey containing MGO were significantly different from those cells treated with 10% (w/v) manuka honey p = <0.001 using a one way ANOVA.
Figure 70: The effect of varying concentrations of manuka honey on the percentage of cells showing septal components: Percentage of MRSA cells with septal components (partial and complete septa) following exposure to manuka honey. EMRSA-15 NCTC 13142 was incubated in nutrient broth (NB) (open diamonds), NB with 10% (w/v) artificial honey (open circles), NB with 10% (w/v) artificial honey+MGO (open triangles), NB with 2.5% (w/v) manuka honey (open squares), NB with 5% (w/v) manuka honey (filled squares), NB with 10% (w/v) manuka honey (filled diamonds), NB with 20% (w/v) manuka honey (filled triangles).
In all images examined cells treated with MGO, like the majority of the cells in other treatments (chapter 4), were entire. The cell surface did not appear to be damaged and so physical evidence of cellular lysis was absent. The cells treated with MGO had no marked increase in the number of septal components seen compared to the control cells $p > 0.05$ (Figure 71).

![Figure 71: Electron micrographs of untreated and artificial honey plus MGO treated S. aureus](image)

Figure 71: Electron micrographs of untreated and artificial honey plus MGO treated S. aureus: The effect of artificial honey containing MGO on the structure of EMRSA-15, NCTC 13142 cells. Electron micrographs show MRSA at 32,000 magnification following incubation for 120 minutes (a) in nutrient broth (NB) alone, (b) in NB containing 10 % (w/v) artificial honey and MGO.

### 7.4.3 2D-Gel Electrophoresis

The initial proteome profiles generated with the use of 2D-gel electrophoresis showed that the methods needed optimising as although clear spot patterns could be seen, there was also a degree of both horizontal and vertical streaking seen (Figures 72a, 72b). Different range IEF strips were used along with increased volt hours at the second dimension stage of electrophoresis to improve the appearance of the gels and reduce the
Figure 72: Example of typical initial 2-D gels of EMRSA-15. Control (top) honey (bottom), horizontal and vertical streaking seen.
streaking seen in the initial profiles. It was demonstrated that increased volt hours with wide range IEF strip produced the clearest profiles (Figure 73a, 73b).

Figure 73: 2-D gels of EMRSA-15 after alterations to processing of gels to optimize spot pattern on gel. Using increased volt hours (a) and narrower range IEF strip (b), horizontal streaking reduced.
All gels were run at least in duplicate to ensure reproducibility (Figure 74). The results for the repeats were similar to the originals; however the quality was better in some than others.

**Figure 74:** Example of the two control 2-D gels of EMRSA-15 used for replicate grouping and spot matching needed for analysis. Two repeats of gels for cellular protein profile of EMRSA-15. Image a) is the original photograph; b) is a repeat.

The analysis of gels was performed using PDQuest Basic 8.0 software. The quality of analysis was slightly problematic, loss of quality of the images was unavoidable as the imaging system captured images in 12 bits and the analysis system needed the images to be in a 16 bit format. The complexity of the analysis software also meant that replicate grouping was used for the master (control) gel but that only the single, clearest gel was analysed for each of the treatments.

The spots from the two control gels (Figure 74) were used as a base line for comparison with all other gels because these were derived from cells growing in media alone, in environmental conditions suitable for the bacteria to grow and reproduce.
The proteome profiles were analysed using the PD Quest software. This initially matched the gels to show how many spots were visible on each gel and how closely the spots on test gels matched those on the replicated control gels. The master control gel of cell treated in TSB alone yielded a count of 243 spots. All the other gels showed a lower spot count indicating a possible down regulation of protein synthesis after treatment (Table 12).

<table>
<thead>
<tr>
<th>Gel Name</th>
<th>Spots counted</th>
<th>Spots Matched to Master gel</th>
<th>Match Rate to Master gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master control</td>
<td>217</td>
<td>217</td>
<td>100%</td>
</tr>
<tr>
<td>Replicate Control</td>
<td>243</td>
<td>242</td>
<td>99%</td>
</tr>
<tr>
<td>Artificial</td>
<td>122</td>
<td>101</td>
<td>82%</td>
</tr>
<tr>
<td>Honey</td>
<td>93</td>
<td>66</td>
<td>70%</td>
</tr>
<tr>
<td>MGO</td>
<td>99</td>
<td>76</td>
<td>76%</td>
</tr>
</tbody>
</table>

**Table 12:** Experiment Summary, showing that most proteins were produced in those cells treated as controls and that the two replicates has a primary spot match similarity of 99%. The total spot counts seen in the other treatment groups were much lower than controls.

The software allowed analysis of the proteome profiles in several slightly different ways. The original experiment summary showed numbers of spots on each profile and its similarity to the master gel (Table 12).

The second type of analysis showed those spots which were unique to each gel i.e. those present on the master but not in the experiment (honey, artificial or MGO), or those present on a test profile but not on the master gel (Figures 75, 76 and 77). Each spot was allocated a SSP number allowing identification of which spots match and which were potentially up
or down regulated proteins. The test gels showed down regulation (fainter or missing spots) of many proteins compared to the master gel (both control gels combined), however although the artificial honey treated profile did not show any up regulation (Figure 75) both the honey treated and the MGO treated test profiles indicated possible proteins that had undergone up regulation compared to the control (Figures 76 and 77).

Figure 75: Examples of 2-D gels proteins expressed in EMRSA-15, 4 hours after treatment with and without artificial honey. Proteins expressed in a) control and not in artificial sample (blue crosses), therefore down regulated by treatment with artificial honey b) Artificial honey treated cells (no up regulated proteins present in this sample, 134 down regulated proteins). Images shown with (bottom image) and without (top image) ssp identity tags.
Figure 76: Examples of 2-D gels proteins expressed in EMRSA-15, 4 hours after treatment with and without manuka honey.  

a) Proteins present in control and not in honey sample (yellow crosses), therefore down regulated by treatment.  
b) Those proteins present in honey treated and not present in the control sample (green crosses) are up regulated (16 up regulated proteins, 157 down regulated proteins). Images shown with (bottom image) and without (top image) ssp identity tags.
These unmatched proteins indicate new proteins being produced, indicating the switching on of protein synthesis. Those present in control gels and not in the treated samples a reduction indicates a switching off of protein synthesis. The protein profile from cells treated with artificial honey was a very different to either the honey or MGO profiles. The artificial honey profile showed no proteins were up regulated. Manuka honey treated and MGO treated cells both showed similar numbers of up regulated proteins at 16 and 18
respectively. It is demonstrated in Table 13 that the proteins switched on in these two treatment groups were not identical.

<table>
<thead>
<tr>
<th>SSP Identity of up regulated honey proteins</th>
<th>SSP Identity of up regulated MGO proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>0406</td>
<td>0406</td>
</tr>
<tr>
<td>0808</td>
<td>0808</td>
</tr>
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<td>4808</td>
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<td>3604</td>
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<td>5902</td>
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<tr>
<td></td>
<td>7604</td>
</tr>
<tr>
<td></td>
<td>0702</td>
</tr>
</tbody>
</table>

Table 13: Examples of proteins in EMRSA-15 up regulated after 4 hours treatment with honey or MGO. Seven of these up regulated proteins were the same (in blue), 9 were unique to the honey treated cells and 11 to the MGO treated cells.

To investigate the identity of those proteins in Table 13 matrix-assisted laser desorption ionisation-time-of-flight mass spectroscopy (MALDI-TOF-MS) would have to be used with an automated spot picker. In this study this technique was not employed due to time constraints and also due to the lack of availability of this equipment at UWIC.

The third type of analysis made possible using the software was the identification of proteins that were present on both master and test profiles but which had undergone a change in expression, either up or down regulation, of more than two fold compared to the
master gel (Figure 78). Different patterns of protein expression are seen on all of the test gels.

Figure 78: 2-D Gels showing those proteins in EMRSA-15 with more than a two fold up or down regulation after 4 hours treatment with or without honey or MGO. Shows proteins marked with coloured symbols with more than a two fold difference in expression, up or down compared to controls. a) Green crosses = all proteins found on master (control) gel, b) red crosses = proteins on artificial gel, c) blue squares = proteins on MGO gel, d) yellow circle = proteins on honey gel.

The proteome profiles were also analysed by comparing them to an already published profile of S. aureus (Kohler et al, 2005). Using the information published here to overlay the profiles generated by the 2-D gel analysis tentative identification of several proteins
was made (Figure 79, Table 14) along with their function in the *S. aureus* life cycle. There was one protein of particular interest shown as blue in
Figure 79: 2-D gel of proteins expressed in EMRSA-15 after 4 hours growth, labelled with presumptive identities. Protein pattern of MRSA-15 control cells grown in TSB after 4 hours. Proteins separated on 2D gels using pH 3-10 strips. Potential spot identities are labelled after comparison with *S. aureus* 2D gel run using very similar method and pH 4-7 strips (Kohler *et al*, 2005).
Table 14. This protein is also indicated on Figure 80 by a circle and it has been preliminarily identified as FabI which is an important protein and possible antimicrobial target.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adk</td>
<td>Adenylate kinase</td>
<td>Purine/pyrimidine metabolism</td>
</tr>
<tr>
<td>CysK</td>
<td>Cysteine synthetase A</td>
<td>Amino acid synthesis</td>
</tr>
<tr>
<td>Dnak</td>
<td>Dnak protein</td>
<td>Protein synthesis, folding and degradation</td>
</tr>
<tr>
<td>Enolase</td>
<td>Enolase</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>FabI</td>
<td>Enoyl-acyl carrier protein reductase</td>
<td>Fatty acid biosynthesis</td>
</tr>
<tr>
<td>Gap</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>GroEL</td>
<td>Chaperonin 60kDa</td>
<td>Protein folding</td>
</tr>
<tr>
<td>MurA</td>
<td>UDP-Nacetylglucosamine 1-carboxyvinyltransferase</td>
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</tr>
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Table 14: Presumptive identity of proteins seen on 2-D gel from EMRSA-15 grown for 4 hours. Proteins of interest identified on Control 2-D Gel (see figure 77). FabI of particular interest shown on gel in Figure 76.
Figure 80: Control 2-D gel (a) of EMRSA-15 protein extraction showing position of FabI protein (known as ssp020, green circle). This protein is not seen in cells treated with honey (Figure 76) or MGO (Figure 77). Figure b same image with ssp identities labelled.
This spot 0201 if it is correctly identified could be a highly interesting protein as it has been identified previously in other studies as the target of antibacterial agents such as triclosan and diazaborines (McMurry et al., 1998; Slater-Radosti et al., 2001).

### 7.5 Discussion

The initial investigation undertaken here looking at time kill data indicates that although MGO does seem to inhibit the growth of MRSA, it is not solely responsible for the effect seen when MRSA cells were treated with manuka honey. This reinforces evidence published by Molan (2008), which suggested that MGO alone does not account for the whole of the UMF factor found in manuka honeys.

Proteomic (2-D gel electrophoresis) analysis of EMRSA-15 was conducted to investigate how honey treatment might affect protein expression within this bacterium. Results presented in previous chapters suggest that the bactericidal effects of honey were not due to physical damage to the cells alone. The increase in septal components observed in Chapter 3, coupled with changes in murein profiles indicted that physiological changes were induced, which were be reflected in the proteomic profile. It was also important to compare the effect of honey with that of artificial honey containing MGO (a potential candidate for the mode of action of manuka honey), and with artificial honey alone to show whether the effects seen are due to the sugars present in honey, hence why MGO is not used without sugar. 2-D electrophoresis is a powerful method of analysing differences in gene expression between two or more groups.
In this instance Wide range 3 - 10 IEF strips were used to maximise the number of proteins detected on the gel. This provided a smaller area in which to resolve the proteins than a narrower pH range strip would have done, but had the advantage of reducing streaking compared to using 4-7 strips. Also it has been reported that protein strips of 4-7 only give a window for the cytoplasmic proteins and that if more alkaline proteins are present they need wider ranging pH strips if they are to be resolved (Hecker et al, 2003) so would produce gels with spots missing. There was still streaking seen on all of the gels. There could be several reasons for the streaking.

Horizontal streaking may be due to excess salt in the samples, which could be removed in future experiments by dialysis or filtration, however the cells were resuspended and disrupted in sterile deionised water in order to keep the salt concentration as low as possible. The streaking could also be due to the presence of nucleic acids or large polysaccharides; these could be removed by treatment of the samples with enzymatic digestion or precipitation with TCA/acetate respectively (Damerval et al, 1986) though all these treatments run the risk of differentially affecting the protein concentrations.

Streaking could also possibly be due to the bonds between protein molecules not being broken. It may be possible to overcome this by carrying out the methylation and reduction steps (equilibration buffers one and two) before the first dimension separation. (Herbert et al, 2001; Galvani et al, 2001; Gorg et al, 1988)

Observations of the gels after staining with Simply blue safe stain gave a high number of visible proteins and it was decided that there was no need to increase the sensitivity of the stain by moving to secondary staining with silver stain; in any case it is not possible to
quantify protein spots that have been stained with silver. It was possible to observe that after all of the treatments that the numbers of proteins were reduced, which is indicative of down regulation of protein synthesis. Since the cells were not being grown in optimum conditions, as were the controls, this was not unexpected. Despite the general decrease in expression of proteins, both the honey and MGO profiles had proteins expressed that were not seen on the control gels. No new proteins were seen after artificial honey treatment. Despite the large amount of data generated by this technique, there are still proteins that will not be picked up by gel based techniques including low abundance proteins and membrane bound proteins (Hecker et al, 2008). The images captured could be improved by having an image capture system that worked in 16 bits to avoid loss of quality when transforming images, also more sensitive stains could be used to pick up small or faint spots missed by the Simply blue safe stain. To improve understanding of how the differing treatments effect MRSA and to identify the separated proteins, the 2D-electrophoresis would ideally have to be coupled to matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. This has been used to look at the effect of antibiotic treatment and oxidative stress on proteins expression in *S. aureus* (Koszczol et al, 2006; Wolf et al, 2008).

The identification of a potential target of manuka honey has been observed as spot 0201, which is seen to be present in control and artificially treated 2-D gels but is missing on both honey and MGO 2-D gels, supporting the idea that MGO is the active component in honey. This protein has provisionally been identified after comparison with previous 2-D gels of *S. aureus* as being FabI (enoyl-acyl carrier protein reductase)(Kohler et al, 2005). If this protein is confirmed as FabI, then it will indicate one mode of action of manuka honey on MRSA. FabI has previously been described as an antibacterial target and it is a selective
target site as it has no mammalian counter part, yet it is ubiquitous in pathogenic bacteria (Bogdanovich et al., 2007). It is involved in the last step of fatty acid biosynthesis and is involved in the assembly of cellular components such as lipopolysaccharides and cell envelope (Park et al., 2007); this could explain why manuka treated cells seen with the electron microscope appear not to be dividing. However it seems logical that there is more than one target or mode of action involved in this process because although the MGO gel also does not show spot 0201, the electron micrographs of MGO treated cells show a different effect compared to manuka honey treated ones.

The analysis of the electron microscopy of MGO treated cells supports the results seen with the proteomic profiling; they show that MGO has a different effect on the formation of septum in S. aureus from treatment with honey. The results show clearly that the effects of MGO treated samples are the same as control cells as regards septal components and different to the honey treated cells. This indicates that if MGO is an active antimicrobial component of manuka honey then it is eliciting its effect in attenuation of the cell cycle than when cells are treated with manuka honey. This compliments the results as reported by Molan, (2008a, 2008b) who show that at low honey concentrations the MGO levels did not correlate with antimicrobial activity. This would indicate that the MGO is acting in synergy with something in the manuka honey which is not found in artificial honey, these results are not entirely unexpected as manuka honey contains high levels of antioxidants and these could decrease the toxicity of MGO to bacteria. For instance the MGO in honey can be consumed by reactions with the hydrogen peroxide or proteins found in honeys (Adams et al., 2009).
The main conclusions from this work were that the treatment of MRSA with manuka caused a difference in the variety of proteins produced when compared to control cells. It has also become apparent that the action of honey does not seem to be caused solely by the presence of MGO, as both treatments down regulated the expression of many proteins and up regulated the expression of a handful of proteins but the up regulation was different for each treatment. Indeed if MGO is having an effect on the viability of MRSA then it is not by the same mode as whole honey which appeared to inhibit the division of the cells. It also confirms that the effects seen on MRSA after treatment with honey are not due to the presence of sugars as although this also caused down regulation of proteins there were no up regulated proteins, this confirms earlier work.

The identities and therefore the significance of the proteins that have been identifies as differentially expressed after treatment can not be determined as yet due as the gel has not been analysed by a MADLI-TOF-MS spot picker and therefore the ssp identities are only qualitative, showing that there are differences in the numbers and intensities of proteins expressed after treatment, but not which proteins these are. However this said if protein 201 was proved to be FabI it would be highly significant as it is essential to convert crotonyl ACP to butyryl ACP, without this step the fatty acid pathway is broken and precursors for the assembly of phospholipids, lipopolysaccharides and the cell envelope would no longer be available leading eventually to cell death, as seen with Triclosan treatment (which targets FabI).
8. Synopsis

Honey was in ancient times used as a first line remedy for numerous ailments, this continued up until the discovery and introduction of antibiotics, which became the mainstream for treatment for infections. Once antibiotic use became the established form of treatment in conventional medicine throughout the world, the use of honey and the knowledge of its beneficial properties diminished and was mostly forgotten until resistance to antibiotics became such that traditional and alternative remedies were once again investigated and used as a solution to the resistance problem.

This work has shown that the mode of action of manuka honey on meticillin-resistant S. aureus is involved in cell division. It has demonstrated that the treatment of MRSA with concentrations above the MIC of manuka honey leads to the inhibition of cell division at some point during or after the septum is formed. There is a clear inhibition of the production/distribution of essential cell division enzyme FtsZ, which could explain the interruption of the cell cycle seen in those cells receiving honey treatment. It has also shown that there is a clear alteration in enzyme and protein production in manuka honey treated cells, summary of results Figure 81. There is some evidence that gene expression is altered by treatment with honey, although there were some problems with reproducibility with the gene expression study and further work would be needed using q-PCR or microarray technology to see to what extent treatment with honey does affect gene expression.

Currently honey is available in several forms for medical use. There has been an increase in the number of products available and honey can be bought in sterilized form and also
Figure 81. Diagram showing a summary of the cell cycle of EMRSA-15 and the possible mechanisms of action of manuka honey which lead to inhibition of the cell cycle and cell death. Also inhibited by manuka honey is the production of FabI which normally occurs throughout the cell cycle. Red crosses indicate the places where the cell cycle is interrupted.
impregnated into varying delivery systems such as gauzes, tulles and plasters. The honeys used within these medical devices are varied but fall into two main categories: Those that derive their action from hydrogen peroxide activity (upon dilution of the honey) and those with a non peroxide antimicrobial activity, the best known of these being the manuka honey which is derived from nectar collected from the *Leptospermum scoparium* shrub in New Zealand. All honeys have some antimicrobial activity normally due to either the hydrogen peroxide activity, high sugar content or low pH (Molan, 1992). The activity in nonperoxide honeys not accounted for by these things has been known as the unique manuka factor (UMF). The extra UMF seen in manuka has also recently been attributed to the presence of methylglyoxal found in manuka honey. It has been shown by Adams et al, (2009) that the MGO itself is created by conversion of dihydroxyacetone to MGO by non enzymatic reactions or by methylglyoxal synthase.

It has been reported that phenolic components can contribute in a small way to the non-peroxide activity of *Leptospermum* honeys (Weston et al, 2000). Recently it has been published that the UMF may be caused by high levels of MGO found in the *Leptospermum* honeys (Adams et al, 2007: Mavric et al, 2008). However, as in the case of the phenolic compounds within honey, MGO alone does not completely explain the non-peroxide activity seen in *Leptospermum* honeys. In a recent review on honey it has been pointed out that considering the MIC given for methylglyoxal, and the levels of methylglyoxal found in manuka honey samples tested (Mavric et al, 2008) this honey would be ineffective at low concentrations. This is clearly not the case as manuka honeys have been shown to have strong antimicrobial activities at low concentrations (Cooper et al, 1999; George and Cutting, 2007). On further examination of the graph published by Adams et al, (2008), correlating antibacterial activity and concentration of methylglyoxal, indicates that the
relationship is linear at higher levels of activity, which would be pure manuka honey, however, extrapolation of this line to zero methylglyoxal leaves a substantial amount of activity unaccounted for by methylglyoxal (Blair, 2008). The claim that non peroxide activity in manuka honeys is entirely caused by MGO needs further investigation. However, it brings about the question of whether if a single component was found to be responsible for the antimicrobial activity seen, should it be isolated and used alone to treat infections or should honey still be used whole. It is likely to be that honey is holistically better used whole than split into its component parts, as has been stated some of the antibacterial activity seen in honey is due to its composition of high osmolarity, low pH and hydrogen peroxide production. The few experiments carried out in this study using MGO show that the effect observed in manuka honey treated cells is not due exclusively to the MGO content within the honey. It has been shown that the structural changes seen in S. aureus cells treated with manuka honey are not seen when treated with artificial honey containing the same levels of MGO as manuka honey. There was no significant difference in septal components seen in those cells treated with MGO compared to control cells, indicating that the effect in manuka honey is caused either only in part by MGO or by MGO in synergy with something else as yet unidentified within the honey. The work done in this study with 2-D gel electrophoresis indicates that it is likely that components within honey work in synergy as MGO alone has a different effect to manuka honey on the cells tested.

The experiments conducted here to explore the effectiveness of honey based wound dressings has shown that they are indeed effective against not only S. aureus but also have some effect on pseudomonads as has been shown previously (Cooper and Molan, 1999; Cooper et al, 2002). It did however show that the effects vary between dressings and that
only a few provide a barrier mechanism which could be useful in a hospital setting to prevent cross infection between patients.

It can be concluded from this work that honey has a complex multi factorial mechanism on inhibition of *S. aureus*, which include a physiological effect in stopping cell division after septal formation but also marked effects on enzyme and protein production. MGO has been shown to play a part in the antibacterial effect of manuka honey however this is most likely working in a synergistic fashion as it does not account for all activity (Molan, 2008a; Molan 2008b). It can also be said that this is probably the best way to continue using honey as no resistance to honey has been reported despite its use over thousands of years. Within our lab we have been unable to create a strain of MRSA resistant to manuka honey. Continuing to use manuka honey as a whole product rather than trying to break it down into its constituent parts would present the best way of making honey a viable alternative in medicine for many years to come.

8.1 Further Work

The work investigated here has looked at the mode of action of manuka honey on MRSA and the results obtained have provided ideas for the future development and advancement of this study.

Given the results seen after the use of confocal microscopy with live/dead staining (Chapter 2) it would be of interest to investigate the likelihood of VBNC cells being present. It could be that the lack of dead cells seen under the confocal microscope and the drop in viable cells in the time to kill is illustrating the adoption of the VBNC state by MRSA. It has been shown that adverse conditions can induce this as a survival strategy in bacteria (Nystrom,
The change in protein synthesis shown in the 2-D work could also be an indicator of reduced culturability in *S. aureus* (downregulation of proteins) as inhibited protein synthesis has been shown to decrease culturability in other species (Reeve *et al*., 1984). It may mean that if different media and growth conditions were employed the total viable counts would have been higher. Typical VBNC response curves have been published showing total counts, culturable counts and viable counts, this method could be used to investigate the response in *S. aureus* (Oliver, 2004).

Further investigation of the observations made in chapters 4, 5 and 6 could be undertaken, to test the effect of MGO for comparison with whole manuka honey. More comprehensive investigation of those various components of regulatory operons, such as *Cid, Lrg* and *Sar* by real time PCR would help indicate if expression is altered by honey treatment. Microarray analysis has been used successfully in rapidly detecting elements of virulence and antibiotic resistance mechanisms, as well as eliciting the effects of varying treatment on transcription of genes (Jaing *et al*., 2008; Jang *et al*., 2008; Riordan *et al*., 2007). Ideally microarray analysis of honey and non honey treated cells could be carried out to enable elucidation of response to honey treatment.

Larger studies into the gold labelling element of the study could be conducted, including earlier time points visualised by TEM of thin sections of cells exposed to honey. The imaging of whole cells would be more efficiently imaged using a scanning electron microscope with back scatter to get a better picture of what is occurring on the extra cellular surfaces of whole cells (Yamada *et al*., 1996). Further work could be conducted utilizing green fluorescent protein reporter gene systems (GFP mutants) to investigate GFP-
FtsZ expression after honey treatment, this GFP system has been used previously to investigate expression and regulation of virulence factors and FtsZ in *S. aureus* and other bacterial models (Pinho and Errington, 2003; Wamel *et al.*, 2002).

It would be interesting to conduct further studies into the effect of honey and its mode of action upon other species of medical importance, such as *P. aeruginosa*. Investigation into the possibility of resistance arising against honey would also be beneficial.

Biofilm formation by pathogenic staphylococci in wounds and on medical devices result in increased antibiotic resistance and are an increasing problem, it would be beneficial to see if honey is effective at preventing or clearing biofilm formation as studies have shown that disruption of regulators in staphylococci can affect biofilm formation (Beenken *et al.*, 2004; Resch *et al.*, 2005; Bayles, 2007) if the mode of action of honey could be more fully elucidated then perhaps it would offer an alternative treatment of biofilm infected wounds, other alternative treatments have already shown promise at eradicating biofilms (Brady *et al.*, 2005).

Ideally further studies into the whole proteome of MRSA could be done, with higher resolution imaging devices, looking at a selection of bacteria over a range of time points to better understand the effects of honey on protein expression within these species. Utilisation of methods such as spot picking and Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDITOF) would allow more accurate investigation of which spots are being up or down regulated by honey, improving understanding of which part of the cell cycle is being affected by various treatments. It would also be beneficial to
investigate the effect of honey on MRSA using microarray technologies, this has previously been used to investigate the effect of honey on *E.coli* (Blair, 2003).

MRSA infections in domestic and farm yard animals have been reported as a problem in their own right (Loeffler *et al.*, 2005; Moodley *et al.*, 2006; van Belkum *et al.*, 2008) it is possible that this could be an area for honey based wound therapy to expand. Studies into the efficacy of honey and honey based wound dressings in this area have already begun with the effect of honey against established biofilms in animal models being investigated (Davis *et al.*, 2008). As well as animal data more *in vivo* data is needed to determine the efficacy of honey in wounds before its acceptance as a main stream first choice treatment.
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Average number of gold particles per cell on whole cell TEM labelling FtsZ.

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**Appendix 2: Gold Labelling Localisation of FtsZ using Transmission Electron Microscopy of Thin Sections.** Average number of gold particles per TEM labelling FtsZ.
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**Appendix 3: Gold labelling Localisation of Endo-B-N-Acetylglucosaminidase using Transmission Electron Microscopy of Whole Cells**  
Average number of gold particles per cell on whole cell TEM labelling Endo-B-N-Acetylglucosaminidase
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Appendix 4: Gold Labelling Localisation of Endo-B-N-Acetylglucosaminidase using Transmission Electron Microscopy of Thin Sections. Average number of gold particles per cell on thin sections cell TEM labelling Endo-B-N-Acetylglucosaminidase