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**Gyamfi-Brobby, George**

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**Signed**

A handwritten signature in black ink, appearing to read 'A. Young', written over a horizontal line.

**Date:** 05/04/2016

# THE MICROBIOLOGY OF DIABETIC FOOT INFECTIONS: A GHANAIAN PERSPECTIVE

**GEORGE GYAMFI-BROBBEY**

A thesis submitted in partial fulfilment of the requirements of the  
University of Westminster for the degree of Doctor of Philosophy

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# Abstract

Diabetic foot ulcer (DFU), a major complication of both types 1 and 2 diabetes, develops in about 15–25% of people living with the disease. In Ghana, DFUs contribute to most hospital admissions (53%) among diabetics with high rates of amputation (33.3%) and death (8.8%). Diabetic foot ulcers are predisposed to infections from bacteria in the environment which normally colonise these wounds as multicellular communities called biofilms. Biofilms have been found to have increased resistance to antimicrobial agents probably due to the presence of an extracellular matrix that retards or prevents the entry of antimicrobial agents into the bacterial community, antibiotic resistance genes and/or the presence of persister cells that are unresponsive to antimicrobial agents.

The work presented here studied the role of 2 multidrug resistant DFU isolates, *Klebsiella pneumoniae* and *Proteus mirabilis* in maintaining the chronicity of diabetic foot ulcers. Using 3 *in vitro* biofilm models; the conventional microtitre plate and Minimum Biofilm Eradication Concentration (MBEC™) High-Throughput assays and the *Quasi-Vivo*® continuous flow system, *K. pneumoniae* and *P. mirabilis* were found to be positive for acyl-homoserine lactone production, biofilm and persister cell producers and could resist and/or tolerate antibiotics such as ceftazidime and levofloxacin up to 1280 times their minimum inhibitory concentration. *K. pneumoniae* and *P. mirabilis* were also found to express the interspecies AI-2 quorum sensing molecules which significantly increased biofilm formation and fold induction of bioluminescence in a *luxS* mutant *V. harveyi* reference strain.

Quorum sensing (QS) inhibition assays using baicalin hydrate, cinnamaldehyde and 2(5H)-furanone showed considerable inhibition of *K. pneumoniae* and *P. mirabilis* biofilm formation but failed to completely inhibit their growth. The combinatorial effects of antibiotics and QS inhibitors/antimicrobial peptides such as polymyxin B and polymyxin B nonapeptide determined as fractional inhibitory concentration (FIC) index suggests that, additive and synergistic effects produced by the combination of two antimicrobial agents have the potential to eradicate biofilms. Data from the FIC indices determined from the combination assays can provide the basis for the formulation of topical treatment for DFUs.

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## List of Presentations

Gyamfi–Brobbeey, G., Greenwell, P., Kimmitt, P. (2012). *Diabetic foot ulcers in Ghana*. Poster presentation at the 2012 Postgraduate Fair, University of Westminster, London, UK

Gyamfi–Brobbeey, G., Greenwell, P., Kimmitt, P. (2013). *Diabetic foot ulcers: prevalence of diabetic foot infections among the diabetic population attending the Komfo Anokye Teaching Hospital, Kumasi, Ghana*. Poster presentation at the 2013 Postgraduate Fair, University of Westminster, London, UK

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## List of Abbreviations

Abbreviation	Full name
μL	micro-Litre
AB medium	Autoinducer Bioassay medium
ACT	Acticoat®
ADEP	Acyldepsipeptide
AHL/HSL	Acyl-homoserine lactone
AI-1	Autoinducer-1
AI-2	Autoinducer-2
AMP	Ampicillin
AMP	Antimicrobial peptide
ANOVA	Analysis of Variance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
ATR	Atrauman
BGC	Biofilm Growth Check
BH	Baicalin Hydrate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BM medium	Basal Modified Medium
BSAC	British Society for Antimicrobial Chemotherapy
CAI-1	<i>V. cholerae</i> autoinducer-1
CAZ	Ceftazidime
CFU	Colony Forming Unit
CIN	Cinnamaldehyde
CIP	Ciprofloxacin
ClpP	Caseinolytic Peptidase
CLSM	Confocal Laser Scanning Microscopy
Con A	<i>Concanavalin A</i>
CPS	Capsular polysaccharide

CV	Crystal Violet
CV %	Coefficient of Variation
DAPI	4',6-diamino-2-phenylindole
DFI	Diabetic Foot Infection
DFU	Diabetic Foot Ulcer
DGGE	Denaturing Gradient Gel Electrophoresis
DM	Diabetes Mellitus
DNA	Deoxyribose Nucleic Acid
eDNA	Extracellular DNA
ELLA	Enzyme-Linked Lectin sorbent Assay
EPS	Extracellular Polymeric Substance
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	Functional Equivalent Pathogroup
FIC	Fractional Inhibitory Concentration
GC-MS	Gas Chromatography – Mass Spectrometry
GDM	Gestational Diabetes Mellitus
GM	Gentamicin
GSS	Ghana Statistics Services
<i>gyrB</i>	Gyrase B
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HAI-1	<i>V. harveyi</i> autoinducer-1
HbA <sub>1c</sub>	Glycated Haemoglobin
HPLC	High Performance Liquid Chromatography
IDF	International Diabetes Federation
JAC	<i>Jacalin</i>
KAN	Kanamycin
KATH	Komfo Anokye Teaching Hospital
Kolliphor® P 407	KP 407
LB	Luria-Bertani
LEV	Levofloxacin

LM	Luria–Marine
LPS	Lipopolysaccharide
MALDI–TOF	Matrix–Assisted Laser Desorption Ionization–Time–of–Flight
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MDA	Medihoney™ Apinate
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mM	milli–Molar
MRSA	Methicillin–resistant <i>Staphylococcus aureus</i>
MTP	Microtitre Plate
MUSCLE	Multiple Sequence Comparison by Log – Expectation
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial and Marine Bacteria
NCTC	National Collection of Type Cultures
NRM	Neutralising Recovery Media
NTG	N–methyl–N'–nitro–N–nitrosoguanidine
OD	Optical Density
OGTT	Oral Glucose Tolerance Test
OPD	Out–Patient Department
PBS	Phosphate Buffered Saline
PE–LB	Protein Extraction & Lysis Buffer
PHA–L	<i>Phaseolus Vulgaris Leucoagglutinin</i>
PIS	Participants' Information Sheet
PMB	Polymyxin B
PMBN	Polymyxin B Nonapeptide
PNA	<i>Peanut Agglutinin</i>
PNP	paranitrophenol–phosphate
QS	Quorum Sensing
QSI	Quorum Sensing inhibitor

RCA-I	<i>Ricinus communis Agglutinin I</i>
RLU	Relative Light Units
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribose Nucleic Acid
SIL	Silvercel®
SJA	<i>Sophora Japonica</i>
SSU	Small subunit
STS	Sodium Thioglycolate Solution
SWA	Sea-water Agar
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAE	Tris-Acetate-Ethylene-diamine-tetra-acetic acid
<i>Taq</i> PCR	<i>Thermus aquaticus</i> Polymerase Chain Reaction
TGGE	Temperature Gradient Gel Electrophoresis
TLC	Thin Layer Chromatography
TSB	Trypticase Soy Broth
UTI	Urinary Tract Infection
UV	Ultra-violet
v/v	Volume/Volume
w/v	Weight/Volume
WHO	World Health organisation
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
ZOI	Zone of Inhibition

# CHAPTER 1

## General Introduction

## **1.1 Diabetes**

Diabetes mellitus (DM) is a chronic disease characterised by high plasma glucose concentrations as a result of the inability of the body to adequately produce or use insulin effectively (International Diabetes Federation, 2015). The disease is diagnosed when there is impaired glucose tolerance and characterised by high plasma glucose concentrations (International Diabetes Federation, 2015). DM has been categorised into three main types (International Diabetes Federation, 2015). Type 1 diabetes mellitus (T1DM) also known as juvenile-onset or insulin-dependent diabetes mellitus is an auto-immune disorder that mostly starts in infected persons before age 40. It is characterised by the self-destruction of insulin-producing beta cells in the pancreas by the body's own immune system. T1DM has been reported in about 10–15% of all diabetes mellitus cases (Rybka, 2010). Type 2 diabetes mellitus (T2DM) formerly known as non-insulin dependent diabetes mellitus is also referred to as late-onset diabetes. The peculiar feature of this type is the relative insulin deficiency and resistance leading to the build-up of plasma glucose (Landon, 2010). This accounts for the bulk of diabetes worldwide of about 90% of all diabetic cases. The third type is gestational diabetes mellitus (GDM) is characterised by intolerance to glucose which is evident during pregnancy after an oral glucose tolerance test (Landon, 2010). Though glucose intolerance can return to normal, pregnant women with family history of diabetes, increasing maternal age, obesity and coming from ethnic group with high risk of developing T2DM can develop permanent diabetes. The babies of such mothers are likely to become obese and have impaired tolerance to glucose (Landon, 2010).

### **1.1.1 Symptoms and Complications of DM**

The major characteristic symptoms of diabetes mellitus result from the abnormal metabolism of glucose leading to high concentrations of glucose in the blood with its attendant complications. This is largely due to either the production of ineffective insulin by a defective pancreas or deficient insulin production. High levels of glucose in the blood otherwise known as hyperglycaemia are as a result of the inability of insulin to transport glucose to the cells for energy and storage. This leads to glycosuria and polyuria which are characterised by the presence of glucose in excreted urine and frequent urination from osmotic imbalance respectively. The body compensates for glucose loss by breaking down protein to release

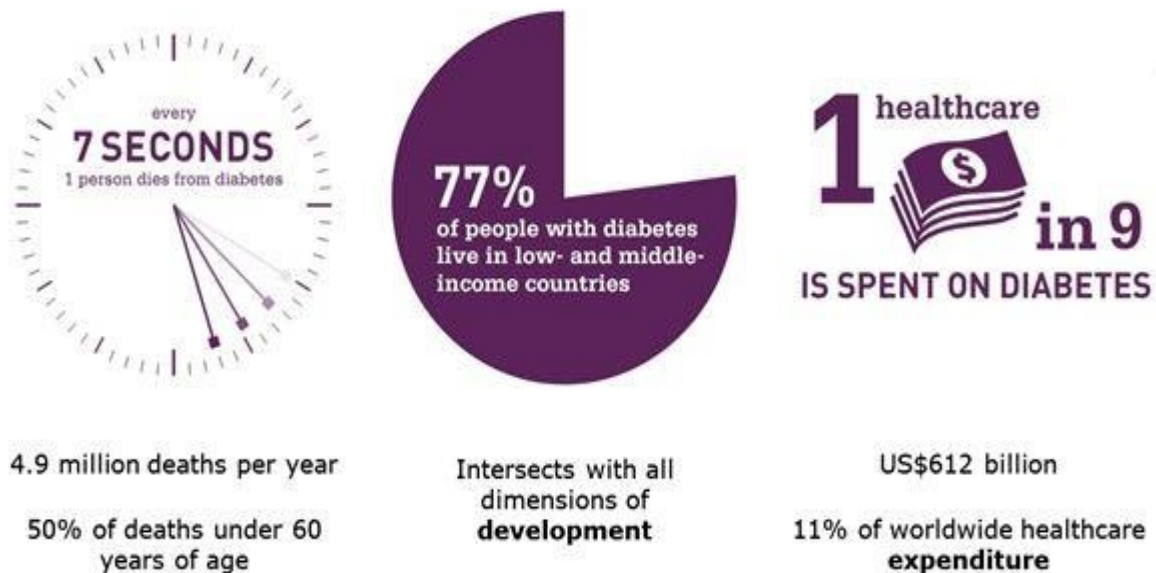
energy in a process called gluconeogenesis. The effect of this process is weight loss as more proteins in the muscles are broken down (Magnusson *et al.*, 1992). Type 2 diabetes mellitus has increased morbidity and mortality rates mostly due to accompanying complications such as neuropathy, nephropathy, retinopathy, microvascular coronary heart disease, cerebrovascular disease and myocardial infarction resulting in cardiovascular and peripheral vascular diseases and diabetic foot ulcers. About 15–25% of diabetic patients will develop a foot ulcer during their lifetime (Singh *et al.*, 2005). Treatment of diabetes has spun from using the extracts of islets of Langerhans from special fish through putting patients on special diet devoid of high carbohydrate contents to transplantation of beta–cells, stem cells and pancreas (Johnson and Luciani, 2010). According to Garcia *et al.* (2001), the successful use of genetic engineering is highly recommended in the treatment of advanced diabetes mellitus in the production of naturally occurring peptides that enable the stimulation of the growth of insulin–producing beta–cells in the pancreas.

### **1.1.2 Global picture of diabetes**

The number of people with the DM is expected to increase from 415 million (8.3% of adult population) to 642 million and beyond by the year 2040 (International Diabetes Federation, 2015). Developing countries will contribute to more than 75% of this increase with a majority of cases among people between 40 and 59 years (International Diabetes Federation, 2015; King *et al.*, 1998; Zimmet *et al.*, 2001). Possible reasons for this increase are due to population growth, ageing, acculturation, obesity and physical inactivity (Wild *et al.*, 2004). Urbanisation, which has been described as the population growth of urban areas due to socio–economic movement of people from less privilege towns and villages to urban centres in search of jobs and good health care has also been found to be associated with lifestyle changes with resultant increases in the number DM cases (Cheema *et al.*, 2014; Ramachandran *et al.*, 2008). Among the 3 types of the disease, type 2 is the commonest and has 7.5% prevalence among the adult population (20 to 79 years). It has been estimated that in addition to the 415 million people living with DM, 318 million adults with impaired glucose tolerance are at high risk of developing the disease (International Diabetes Federation, 2015). This is because increase in the prevalence of the disease is directly related to increase in cultural and societal changes. For example, in high income countries, T2DM accounts for up to 91% of the adult population living with the disease (Largay, 2012). The

number of people with undiagnosed diabetes who risk the development of sudden complications has been estimated as 193 million worldwide (International Diabetes Federation, 2015). Figure 1.1 illustrates the current global picture of the disease.

As a result of genetic predisposition and social deprivation diabetes tends to cluster in some ethnic groups such as people of South Asian and African lineage (Chen *et al.*, 2012; Diabetes in the UK, 2012). Complex combinations of genetic and environmental factors as well as social deprivation make DM about six times more common among people of South Asia descent and about three times more common in people of African and African–Caribbean lineage (Chen *et al.*, 2012).



**Figure 1.1** Global burden of DM (Image Source: International Diabetes Federation, 2014).

### 1.1.3 Diabetes in Ghana

Diabetes prevalence in Ghana was first established as 0.4% in 1958 (Dodu, 1958). The current operational prevalence, 6.3% was established by Amoah *et al.* (2002). This prevalence is expected to increase to 11.0% by the year 2035 in line with predictions by the World Health Organisation despite increased awareness of the disease (Amoah *et al.*, 2002; King *et al.*, 1998). This implies an annual increase of 35.8% of newly diagnosed cases with associated complications. The predicted increase of 4.7% in the national prevalence of DM can also be attributed to an increase in education and awareness of the disease as well as



better systems of screening and recording of new cases. Studies on diabetes in most African countries including Ghana have mainly focused on the use of biochemical assays such as oral glucose tolerance test (OGTT) to determine plasma glucose concentrations, and glycated haemoglobin or haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) test to estimate the amount of bound glucose to red blood cells in establishing the presence and severity of the disease (Amoah *et al.*, 2002; Levitt, 2012; Lehman, 2009). Glycated haemoglobin has been defined as the irreversible glycosylation of haemoglobin A<sub>1c</sub> at either one or both N-terminals (Syed, 2011). The covalent bond that leads to the glycosylation of haemoglobin A<sub>1c</sub> remains throughout the lifespan of the bound red blood cells (Syed, 2011). Hence, the estimation of HbA<sub>1c</sub> plasma concentration has been used as an indicator of glucose concentration throughout the lifespan (120 days) of red blood cells (Kobold *et al.*, 1997; Khaw *et al.*, 2001; McCane *et al.*, 1994). HbA<sub>1c</sub> has therefore been employed as a diagnostic tool to monitor the control of blood glucose in diabetic patients (Kobold *et al.*, 1997; McCane *et al.*, 1994). In addition, foot infection is confirmed by physical examination of the foot and the estimation of increased total white blood cell count associated with increased body temperature (Amoah *et al.*, 2002). In some developing countries including Ghana, it has been found that diabetic foot ulcers contribute to most hospital admissions (Asumanu *et al.*, 2010; Kengne *et al.*, 2006; Ogbera *et al.*, 2006; Singh *et al.*, 2005). Asumanu *et al.* (2010) studied the impact of diabetes and foot ulcers on patients admitted at the 37 Military Hospital, Accra, Ghana and reported that 53% of people admitted with diabetes had foot ulcers, with 33.3 and 8.8% resulting in amputations and death respectively. However, health policies have over the years placed little emphasis on the effective control and prevention of diabetes. In Ghana, treatment for diabetic foot infections (DFI) involves the administration of broad-spectrum antibiotics before or after the request for a wound swab for bacterial culture and antibiogram according records documented at the Microbiology department, Komfo Anokye Teaching Hospital, Kumasi, Ghana. Most commonly used antibiotics include the quinolones such as ciprofloxacin and levofloxacin, and third generation cephalosporins such as cefotaxime and ceftazidime. Other antibiotics used include metronidazole, clindamycin and aminoglycosides such as gentamicin and tobramycin (Bonham, 2001; Nelson *et al.*, 2006; O'Meara *et al.*, 2000). Other treatment options include topical applications of biocides such as 0.25% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and antimicrobial agents such as honey (Ankra-Badu, 1992; Molan, 2006; Zumla and Lulat, 1989). H<sub>2</sub>O<sub>2</sub> at low concentrations has been found to act as a signal molecule that attracts

leucocytes leading to the production of cytokines and chemokines at the wound site during the inflammation phase of wound healing (Schreml *et al.*, 2011). In addition, low concentrations (at 10 mM) of H<sub>2</sub>O<sub>2</sub> have been reported to promote connective tissue formation and enhance wound closure (Loo *et al.*, 2012). On the other hand, high concentrations (at 166 mM) of H<sub>2</sub>O<sub>2</sub> have been associated with oxidative and nitrative damage of lipid and protein, reduced connective tissue formation and subsequently prevent wound closure and healing (Loo *et al.*, 2012). The catalysis of H<sub>2</sub>O<sub>2</sub> has been found to produce hydroxyl free radicals which destroy bacterial cell components such as proteins, lipids and DNA by attaching to exposed sulfhydryl groups and double bonds (McDonnell and Russell, 1999; Schreml *et al.*, 2011). Honey has been a traditional salve for wounds for centuries and its antimicrobial activities include antioxidant activity, anti-inflammatory action, debriding action, enhanced rate of wound repair and deodorising action (Seckam and Cooper, 2013). As an antioxidant, topically applied honey has been reported to scavenge reactive oxygen species produced during tissue inflammation, enhance connective tissue formation and granulation, wound closure and wound healing (Henriques *et al.*, 2006; Seckam and Cooper, 2013; Subrahmanyam *et al.*, 2003).

#### **1.1.4 Diabetic foot ulcers (DFU)**

Diabetic foot and the associated consequences are known to have a global economic cost of billions of dollars with respect to treatment, lengthy hospital stay and subsequent amputation (Frykberg *et al.*, 2000; Reiber *et al.*, 1995). Complex combinations of predisposing factors are largely responsible for ulceration and amputation. The formation of ulcer from trauma and or excessive pressure on deformed foot is mostly as a result of neuropathy with sensory, motor and autonomic disturbances of the nervous function. Others include neuro-osteoarthropathic deformities (Charcot disease), vascular insufficiency, hyperglycaemia and other metabolic derangements, patient disabilities, maladaptive patient behaviours, trauma and healthcare system failures (Caputo *et al.*, 1994; Frykberg, 1998). These factors result in a number of mechanisms from abnormal foot anatomy and biomechanics, lack of protective sensation, deficient sweating through impaired immunological function and wound healing, reduced vision, limited mobility, poor compliance with medical care, excessive weight-bearing to inadequate patient education and monitoring of glucose control and foot care eventually leading to injury or impairment (Caputo *et al.*, 1994; Frykberg, 1998). Lipsky *et*

*al.* (2004) defined DFU as any inframalleolar infection in an individual with diabetes mellitus. Examples of inframalleolar infections are cellulitis, myositis, abscesses, necrotising fasciitis, septic arthritis, tendonitis, osteomyelitis and paronychia.

### 1.1.5 Diabetic foot infection (DFI)

According to Lipsky *et al.* (2004) the infected diabetic foot mal perforans ulceration is a prominent and typical lesion (Figure 1.2). The presence of an ulcer increases the susceptibility of the wound to infection from bacteria. An active infection progresses from bacterial colonisation through contiguous extension involving deeper tissues to limb-threatening infection beyond 2 cm from the ulcer perimeter (Frykberg, 2002; Lipsky *et al.*, 2004). In most cases increase risk and severity of foot infections are as a result of poorly characterised immunologic disturbances, poor blood circulation or impaired tissue perfusion (Geerlings and Hoepelman, 1999). Limb-threatening infections with subsequent amputations are normally characterised by deep tissue abscess with severe cellulitis and osteomyelitis in critically ischaemic limbs (American Diabetes Association, 1999). Signs indicative of infection include purulence and inflammation and these should usually be followed by aerobic and anaerobic cultures. Purulent drainage or curetted materials from infected ulcers provide the best specimen for bacterial culture.



**Figure 1.2** Diabetic foot presentations with ulcerations on the; (A) the plantar and (B) the dorsum of the forefoot (Image source: <http://worldlatest.net/files/2015/03/3-112>).

## 1.2 Biofilms in Diabetic Foot Infections

Despite the documentation of biofilms more than a century ago by Anthony van Leeuwenhoek, their clinical importance became evident when they were indicated in chronic infections (Bispo *et al.*, 2015; Potera, 1999). In the preface of her book titled ‘*Control of Biofilm Infections by Signal Manipulation. Springer Series on Biofilms 2*’, Balaban (2008) suggested that there may be more biofilm-related diseases than the total numbers affected by heart disease and cancer. Ever since Akiyama *et al.* (1993) and Serralta *et al.* (2001) demonstrated the presence and nature of biofilms in *in vitro* and in experimental murine and pig models through staining and microscopy, more researches has been conducted on biofilms to increase understanding of biofilms (Cooper, 2010; Lopez *et al.*, 2010). Although there still remain some challenges in biofilm research such as the diagnosis of biofilm infection and bacterial culture, biofilm infection and colonisation, and the issue of antibiotic susceptibility of planktonic cultures compared to biofilm resistance to antibiotics and/or the presence of antibiotic tolerant persister cells, the study of biofilm is one of the most relevant topics in wound biology (Cooper, 2010; Hall–Stoodley and Stoodley, 2009; James *et al.*, 2008; Lopez *et al.*, 2010; Mertz, 2003; Wolcott *et al.*, 2008). It has therefore been suggested that the impairment of healing among people with chronic wounds including diabetic foot ulcers may be due to biofilm phenotype infections (James *et al.*, 2008; Wolcott *et al.*, 2008).

### 1.2.1 The Nature of Biofilms

Biofilms have been described as the ubiquitous and natural phenotype of bacteria (Cooper, 2010; Lopez *et al.*, 2010). Costerton *et al.* (1999) defined biofilm as ‘a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.’ According to Davey and O’Toole (2000) and Donlan (2002), the extracellular polymeric substance (EPS) can be considered as the primary matrix matter that accounts for about 50–90% of the biofilm. The chemical composition of the EPS may vary depending on the bacteria present in the biofilm but is primarily made up of polysaccharides (Donlan, 2002). For example, in the case of Gram-negative bacteria polysaccharides present in the EPS are neutral or polyanionic which confers anionic properties to the EPS (Sutherland, 2001). Some of these anionic polysaccharides include D-galacturonic, D-glucuronic, and mannuronic acids as well as ketal-linked pyruvates (Sutherland, 2001). Other components of

the EPS such as proteins and extracellular DNA, together with the various types of polysaccharides are known to provide structural support for the growing biofilm (Lopez *et al.*, 2010). In *Pseudomonas aeruginosa*, 3 genes loci designated as alginate (*alg*), pellicle (*pel*) formation and polysaccharide synthesis locus (*psl*) genes have been found to produce 3 exopolysaccharides namely; alginate, PEL and PSL (Branda *et al.*, 2005; Colvin *et al.*, 2012; Jackson *et al.*, 2004; Matsukawa *et al.*, 2004). In *Staphylococcus aureus* biofilms, biofilm-associated proteins called *Bap* maintain the structural integrity. Other proteins that support the biofilm structure are fimbriae (Latasa *et al.*, 2006). In addition, *P. aeruginosa* and *S. aureus* also produce extracellular DNA called eDNA that provides stability to the biofilms (Lopez *et al.*, 2010; Rice *et al.*, 2007). As a result of their compositions, biofilms have also been referred to as microbial communities containing microcolonies of bacterial cells enclosed in extracellular polymeric substances separated from each other by interstitial voids (Lewandowski, 2000). Stoodley *et al.* (1997) have also suggested that biofilms could be described as thin-based films which range from being sparse and sporadic monolayers of cells up to some cell layers in thickness. The amount of EPS and the total thickness of biofilm produced by biofilm-producing bacterial species normally vary between different bacterial species, shear forces experienced by the bacteria and other environmental conditions such as oxygen concentration, temperature, pH and availability of nutrients (Flemming and Wingender, 2010). It has been shown that cell-to-cell (between bacterial cells or bacteria and host cells) signalling play a possible role in early attachment and detachment of bacteria to surfaces during biofilm formation (De Kievit *et al.*, 2001; Xie *et al.*, 2000). Donlan and Costerton (2002) also added that biofilms further show evidence of altered phenotype with respect to growth, gene expression and protein production. The evidence of altered phenotype is as a result of communication among the bacteria in this sessile community referred to as quorum sensing. Quorum sensing will be discussed later in the literature review of this thesis (see section 1.4).

Microorganisms are ubiquitous, and their distribution and dimensional movement in the environment are usually determined by chemical conditions like nutrient availability, the concentrations of inhibitory substances in addition to chemical factors such as oxygen tension, temperature or acidity, and biological factors such as predation and competition (Cooper, 2010; Mitchell and Kogure, 2006; Watnick and Kolter, 2000). The short supply of nutrients most often limits bacterial growth in their natural habitat. Therefore, planktonic

cells are attracted by complex concentration gradients created by nutrients adsorbed onto inert surfaces. The initial attachment of bacteria onto a surface (inert or living) prior to biofilm formation depends on factors that include the types of surface (substratum), formation of conditioning film on the surface, aqueous medium and its hydrodynamics and characteristics, and properties of the cell surface (Donlan, 2002). The ideal environment suitable for attachment and subsequent growth of bacteria is a solid–liquid interface (O’Toole *et al.*, 2000). It has also been established that, rough surfaces with higher surface area have diminished shear forces exerted by the surrounding medium which in turn increase the rate and extent of bacterial attachment onto that surface (Characklis *et al.*, 1990). Physicochemical properties such as hydrophobicity rather than hydrophilicity of material surfaces such as the wells of 96–well plates and Teflon has also been found to increase the rate of bacterial attachment (Bendinger *et al.*, 1993; Fletcher and Loeb, 1979; Pringle and Fletcher, 1983). Hydrophobic interactions that take place between bacterial cell surfaces and the substratum of the material surface have been found to enable the bacterial cells to irreversibly attach to the surface after overcoming active repulsive forces that are present (Pringle and Fletcher, 1983).

Prior to the irreversible attachment of bacterial cells onto a surface (either abiotic or biological) in an aqueous medium, the surface is coated by polymers from the medium called conditioning film in order to overcome active repulsive forces that prevent attachment (Loeb and Neihof, 1975). The nature of conditioning films exposed for attachment on biological (*in vivo*) and abiotic (*in vitro*) surfaces has been found to be differ in response to the media from which they are produced (Donlan, 2002). Human host conditioning film – producing media include tears, urine, blood, saliva, respiratory secretions and intervascular fluid (Mittelman, 1996). They have been found to alter characteristics, such as hydrophobicity or hydrophilicity of the substratum (Ofek and Doyle, 1994). Conditioning films also known as microbial surface components recognising adhesive matrix molecules (MSCRAMMs) produced in human hosts which facilitate bacterial attachment to biomaterials or host surfaces comprise of glycoproteins, lipids, phosphoproteins, lysozymes and albumin (Clarke and Foster, 2006; Foster and Höök, 1998; Marsh, 1995). The effect of conditioning film on surfaces causes changes in the hydrodynamic interactions between the bacterial cells and the surface leading to decreased flow velocities and shear forces with resultant increase in the rate of microbial attachment (Rijnaarts *et al.*, 1993; Zheng *et al.*, 1994). According to O’Toole and Kolter

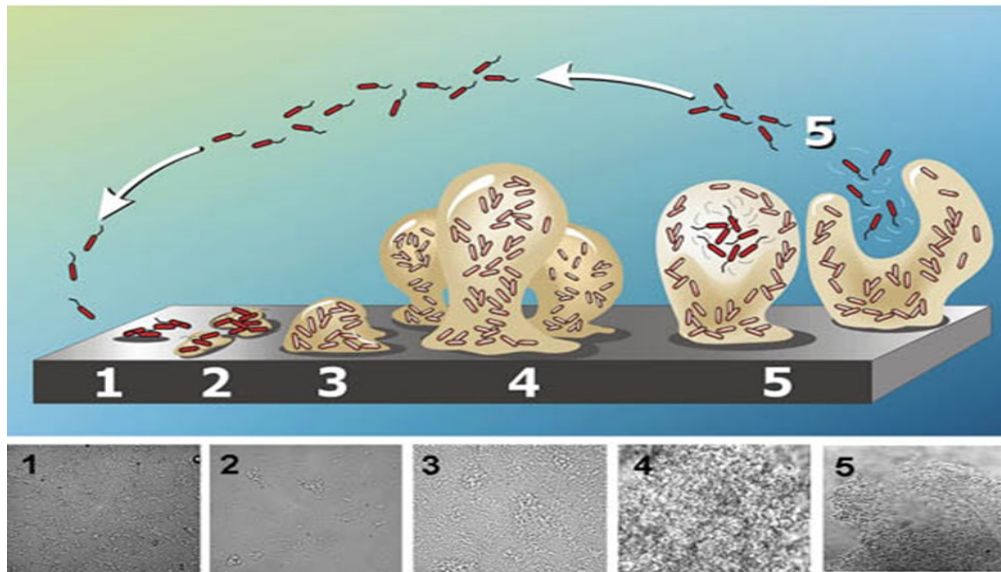
(1998), individual *P. aeruginosa* cells that initiate biofilm development on an abiotic surface adhere to these surfaces with small amounts of EPS (Stage 1, Figure 1.3). During the stage of reversible attachment, bacterial cells are capable of moving independently in a twitching or gliding manner by means of their cell surface appendages called pili (O'Toole and Kolter, 1998; O'Toole *et al.*, 2000). As a result of this independent movement, some bacteria may leave the surface to colonise new surfaces as planktonic cells while the remaining adherent cells may continue with the processes of biofilm formation (Stoodley *et al.*, 2002). Prior to differentiation of adherent cells, the bacterial cells have been found to demonstrate some species-specific behaviours such as rolling, creeping, windrow-like formation and the subsequent formation of aggregates (Marshall *et al.*, 1971; Korber *et al.*, 1995). In chronic wounds, the initiation of irreversible attachment onto the wound bed sometimes occurs when planktonic bacteria sense environmental cues such as change in pH, salinity, quorum sensing molecules and nutrients (Wolcott *et al.*, 2008). Consequently, cell-surface adhesins attach and bind to specific host cell epitopes in collagen, fibronectin, fibrinogen and vitronectin (Foster and Höök., 1998; Massey *et al.*, 2002). Davies and Geesey have demonstrated that initial attachment of *P. aeruginosa* onto a surface triggers the upregulation of genes responsible for the production of alginate which further strengthens the bacteria-host attachment. In the case of *P. aeruginosa*, the formation of an irreversible attachment with host cells results in a metabolic shift that directs the expression of biofilm phenotypes (Harrison-Balestra *et al.*, 2003). Once a permanent attachment has been achieved, the attached bacterial cells divide into biofilm phenotypes which begin to secrete the EPS (Stage 2, Figure 1.3). As the cells continue to grow and develop into mature biofilms, they form microcolonies separated by water channels (Stage 3, Figure 1.3). The structure of the microcolonies is held together by the EPS which is primarily made up of proteins, polysaccharides and nucleic acid as well as organic acid that serve as building blocks to hold the cells together. As the biofilms mature, some individual microcolonies detach from the surface (with or without apparent perturbation) and disperse giving rise to planktonic cells that colonise new areas (Stages 4 and 5, Figure 1.3).

Costerton *et al.* (1995) described the architecture of the microcolonies of mature biofilms as 'discrete pillar- or mushroom-shaped structures' connected by a complex extracellular matrix that provides access to nutrients. Biofilms have a number of advantages over free-living microbes. The EPS is able to capture and concentrate carbon, nitrogen and phosphate

as nutrients for the growing biofilm (Beveridge *et al.*, 1997). The ability of biofilms to resist the action of removal agents notably antibiotics, host phagocytic clearance and host oxygen radical and protease defences has also been reported (O'Toole *et al.*, 2000; Wolcott *et al.*, 2008). A study by Liu and Tay (2001) has demonstrated that biofilms could withstand detachment from varying degrees of shear stresses on abiotic surfaces by regulating their metabolic pathways (anabolism and catabolism) in response to substrate flux. The biofilm bioreactor experiments by Liu and Tay (2001) have suggested that shear stresses of flow velocities between 0.48 and 1.45 m/s, lead to the formation of biofilms of various thickness with higher flow velocity resulting in thinner and denser biofilm with a compact structure. Together with other reports, Liu and Tay (2001) have suggested that biofilm consortia could regulate their metabolic pathways in order to maintain a balance with an external detachment force by making use of non-growth energy that eventually decreases net negative charge, increases hydrophobicity and induces dehydration of the cell surface leading to increased cell-cell attachment and the formation of a denser biofilm consortia (Chen *et al.*, 1998; Low *et al.*, 2000; Marshall and Gruickshank 1973; Pringle and Fletcher 1983; Russell and Cook, 1995; van Loosdrecht *et al.*, 1987). Lastly, mature biofilms have the potential to detach from the growing matrix by means of dispersion as a result of mechanical shear pressure or through a programmed response engineered genetically (Boyd and Chakrabarty, 1994). The detached biofilm is capable colonising fresh surfaces and exhibit all the characteristics of the parent biofilm such as resistance to antimicrobials and host immune responses (Shirtiliff *et al.*, 2002).

Biofilms have often been associated with human conditions involving the indwelling of medical devices and prosthetics such as prosthetic joints, heart valves, contact lenses, catheters, stents and intrauterine implants (Adal and Farr, 1996; Archibald and Gaynes, 1997; Dickinson and Bisno, 1993; O'Toole *et al.*, 2000). Although mixed species biofilm has been found to be predominant in most environments, the presence of single-species biofilms has also been reported in other infections and on surfaces of medical implants such as catheters and prosthetics (Adal and Farr, 1996; Cooper, 2010; Dickinson and Bisno, 1993; O'Toole *et al.*, 2000). Bacteria that most often cause infections in these conditions include coagulase – negative *Staphylococci*, *P. aeruginosa* and sometimes Gram-negative bacilli and *Candida*. The cause of such infections might be as a result of the use of contaminated devices prior to surgery (Cooper, 2010). Treatment is most often by the removal of the associated implant.





**Figure 1.3** A model and electron micrograph illustrating the 5 stages of *P. aeruginosa* biofilm growth cycle. Stage 1; planktonic cells attach to a surface; Stage 2 – cells divide, secrete more EPS and form microcolonies; Stage 3 – developed microcolonies separated by water channels; Stages 4 and 5 – mature biofilms with “mushroom-like structures”, detach and disperse planktonic cells to colonise new areas (Stoodley *et al.*, 2002).

### 1.2.2 Concept of Functional Equivalent Pathogroups (FEP)

The proposers of the concept of FEP, Dowd *et al.* (2008b) argued that the co-morbidities connected with the pathophysiology of most chronic wounds need to be defined in context of the host conditions. This they believe is an important step prior to the management of chronic wound. They further argued that the ecology of a wound comprise of both host conditions and host’s environment. This means that some patients may share a similar ecological wound as a result of similar environmental conditions and hosts’ conditions. Dowd *et al.* (2008b) studied 8 major clusters of bacterial isolated from various wounds including DFU and named them functional equivalent pathogroups. They hypothesised that the chronicity of infectious wounds cannot be maintained by some bacterial species on their own unless they co-occur in significant mixtures in order to symbiotically act to establish pathogenicity, develop biofilm and maintain the chronicity of the wounds.

The occurrence of pathogroups in wounds especially diabetic foot infections cannot be easily identified using traditional culturing methods (Dowd *et al.*, 2008b). This is because almost all FEPs are linked together by anaerobes which are difficult to isolate by traditional culture

techniques. Some studies have shown that some biofilms made up of both aerobes and anaerobes have the ability to withstand certain stressors by a process called coaggregation (Bradshaw *et al.*, 1998). A model biofilm created by the Lewandowski laboratory has suggested that only bacteria found on the surface of biofilms use oxygen suggesting that internal regions of the biofilm are occupied by facultative and obligate anaerobes (Rasmussen and Lewandowski, 1998). Although the biofilm model described by Rasmussen and Lewandowski (1998) may not be applicable to all biofilm settings, the complex and polymicrobial nature of biofilms require that sensitive methods are used in their identification. Though the cultivability of bacterial pathogens has been the central practice in medical microbiology, the need to incorporate molecular technology in the attempt to deal with obstinate infections such as polymicrobial and complex infections has become clear (Dowd *et al.*, 2008b).

### **1.2.3 Detection of biofilms in wounds**

It is estimated that biofilm–infected foot ulcers make up 85% of lower limb amputations among diabetic patients (Adler *et al.*, 1999). In 2005, the National Diabetes Information clearinghouse (USA) estimated that at least 80,000 amputations are performed each year among the diabetic population in United States of America. As mentioned earlier, diabetes–related amputations are mostly preceded by wound infections, impaired wound healing and ischaemia in conjunction with a foot ulcer. One of the earliest discoveries of biofilms in wounds was observed using scanning electron microscopy of 15 sutures and 15 staples in healed surgical wounds (Gristina *et al.*, 1985). *Staphylococcus epidermidis* was isolated from specimens obtained from the sutures. Surprisingly, the observed biofilms had neither triggered any obvious infection nor immunological host response. The formations of biofilms have been successfully demonstrated and examined using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) in acute wounds using murine and pig models respectively (Akiyama *et al.*, 1993; 1994, Serralta *et al.*, 2001). Akiyama *et al.* (1996) demonstrated the formation of glycocalyx, a thick layer of extracellular polymeric substance by *S. aureus* in a biofilm. Serralta *et al.* (2001) also demonstrated the production of a polymeric matrix encasing *P. aeruginosa*.

Apart from the use of advanced microscopy mentioned above, the presence of biofilms in chronic wounds has been demonstrated by molecular characterisation of the microbiota found in pooled biopsy specimens using ribosomal RNA gene analysis techniques. In one such study, Dowd *et al.* (2008a) demonstrated the dominance of *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia* and *Serratia* species in chronic wounds using pyrosequencing and full ribosome shotgun sequencing. In another study, discrete biopsy samples from 40 diabetic foot ulcer patients were analysed by bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *Corynebacterium* species was predominant followed by the presence of some obligate anaerobes including *Bacteriodes*, *Peptoniphilus*, *Finegoldia*, *Anaerococcus* and *Peptostreptococcus* species (Dowd *et al.*, 2008b).

The biosynthesis of the EPS is a major aspect of the development of a mature biofilm. The presence and classes of polymeric substances in the EPS varies with every bacterial cell. However, the main macromolecules found in the EPS include polysaccharides, proteins, DNA, glycoconjugates such as glycoproteins and glycolipids, and phospholipids (Czacyk and Myszka, 2007; Branda *et al.*, 2005; Sutherland, 2001). For example, EPS components such as alginate produced by *P. aeruginosa* can be used as a marker for biofilm detection in cultures, or on medical devices such as catheters by means of scanning electron microscopy examination, staining and examination with light microscope or epifluorescence microscopy (Davies *et al.*, 2007; Harrison–Balestra *et al.*, 2003; Stickler *et al.*, 1998). It has been suggested that some extracellular molecules (polysaccharides, glycoproteins and proteins) synthesised as part of the EPS in some bacteria have antigenic properties that can be used for serological classification of these bacterial cells (Branda *et al.*, 2005; Czacyk and Myszka, 2007; Sutherland, 2001). Czacyk and Myszka (2007) also mentioned that physiological determinants such as pH of culture medium, carbon/nitrogen availability, incubation temperature and growth phase influence EPS biosynthesis.

The estimation of biofilm formation using the conventional microtitre plate method is one of the most widely used *in vitro* assays for the determination of biofilm formation. The microtitre plate assay provides the medium for biofilm-forming bacterial species to adhere and form biofilm in a batch culture which can be detected and estimated through staining with stains like safranin or crystal violet (Christensen *et al.*, 1985; Merritt *et al.*, 2011).

Although the morphology of the biofilm cannot be seen, the ability of organisms to form biofilm can easily be determined in a simple and reproducible analysis. In batch culture using 6-well or 24-well plates or in a continuous flow cultures, biofilms can be grown on slides, stained and examined by light, epifluorescence or confocal laser microscopy. Viable bacterial cell counts can also be determined after biofilm batch cultures (Sun *et al.*, 2008).

Further detection of biofilms in wounds known as the reporter assay was developed in rats by Nakagami *et al.* (2008). This assay involved the comparison of the levels of quorum sensing molecules detected in 12 pressure-induced ischaemic wounds infected with *P. aeruginosa* to 12 uninfected wounds. The reporter assay test recorded change of colour in a bacterial culture containing acyl-homoserine lactone quorum sensing molecules. Other methods of detecting quorum sensing molecules by means of chemical analysis have been developed. They include mass spectrometry, thin layer chromatography and high performance liquid chromatography (HPLC). However, all these tests are limited to research laboratories and cannot be performed as routine tests in hospitals.

#### **1.2.4 Control of biofilms**

From section 1.2.3, it can be established that there is a link between wound chronicity and the presence of biofilms. It is also evident that the presence of antibiotic resistant strains contributes significantly to treatment failure (Noiby *et al.*, 2010). There is therefore the need to devise strategies in controlling such wounds and reduce the need for amputations.

Control measures required to combat biofilms can either be preventive or those that remove and interrupt their formation. One such strategy described by Wolcott and Rhoads, (2008) in the treatment of critical limb ischaemia involved sharp wound debridement and the administration of lactoferrin and xylitol to disrupt the formation of the extracellular polymeric substance by removing iron. Other remedies suggested include; biological debridement with maggots (van der Plas *et al.*, 2008), enzymatic disruption of EPS (Johansen *et al.*, 1997), use of antimicrobial agents such as cadexomer, iodine, hydrogen peroxide, chlorohexidine, octenidine, polyhexanide, and silver (Akiyama *et al.*, 2004; Johansen *et al.*, 1997; Percival *et al.*, 2007). In another study, it was shown that the sugar content in honey has the ability to prevent the adherence of *P. aeruginosa* to host cells in the development of infection (Lerrer *et al.*, 2007). Fructose and fucose carbohydrate monomers found in honey

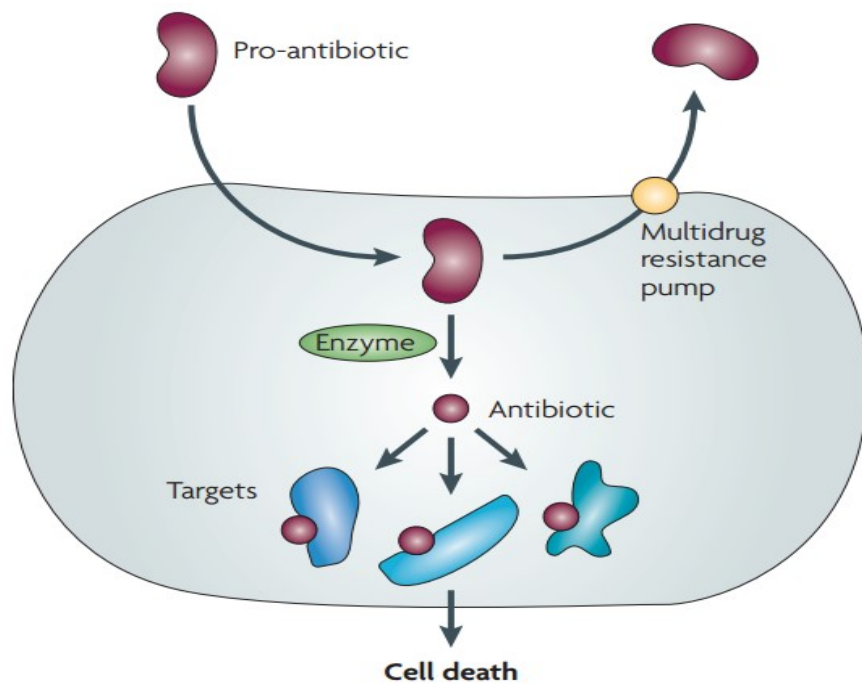
have been demonstrated to block sugar – binding proteins, also called lectins, on the surface of *P. aeruginosa* preventing them from attaching to target cells and inhibiting biofilm formation (Merckoll *et al.*, 2009; Tielker *et al.*, 2005).

### **1.3 Resistance of biofilm to antibiotics/antimicrobial agents**

Clinically important biofilms can normally be formed in diseased conditions such as endocarditis and diabetic foot ulcers in response to stresses from host defence mechanisms and antimicrobial treatment (Anderson and O’Toole, 2008; Mah and O’Toole, 2001; Parsek and Singh, 2003). According to Lewis (2007), an antibiotic is initially administered into a host in its benign form (pro-antibiotic) after which it is enzymatically converted into an active form in the cytoplasm. The active form of the antibiotic then binds to its target site and causes the needed conformational changes that lead to cell death (Lewis, 2007). Biofilms have been found to have increased resistance to antimicrobials and sustained host defence mechanisms than planktonic cells (Lopez *et al.*, 2010). The ability of bacteria to resist host defence mechanisms has been linked to their ability to interact with each other through cell–cell adhesion using cell–surface appendages, quorum sensing (bacterial communication) and the presence of antibiotic resistant and/or tolerant (persister) cells which subsequently lead to treatment failure (Bigger, 1944; Costerton *et al.*, 1999; Donlan and Costerton, 2002; Dowd *et al.*, 2008a; Dowd *et al.*, 2008b; James *et al.*, 2008, Wolcott *et al.*, 2008; Lewis, 2005; Lewis 2007; Lewis 2010; Conlan *et al.*, 2013; Tielker *et al.*, 2005; Winzer *et al.*, 2000). Bacterial cell–cell interactions and quorum sensing have been further discussed in chapter 5, sections 5.1 and 5.7, and throughout Chapter 5. In most cases, the mechanisms fundamental to antibiotic resistance are due to target modification by mutation; enzymatic changes leading to target modification; substitution of target (expression of alternative target); modification or destruction of antibiotic; antibiotic efflux and restricted entry of antibiotics (Lewis, 2007).

In biofilms, the EPS acts as a physical barrier that inhibits the penetration of large antimicrobial proteins such as lysozyme and complement from the host immune system (Bjarnsholt *et al.*, 2005; Lewis, 2001). Moreover, the negatively charged extracellular polymers within the matrix are able to bind to positively charged antibiotics and antimicrobial peptides such as the aminoglycosides (gentamicin and tobramycin) and defensins and prevent their subsequent permeation (Bagge *et al.*, 2004; Brodgen, 2005;

Shigeta *et al.*, 1997). Antibiotic-degrading bacterial enzymes such as N-acetyltransferases, O-nucleotidyltransferases and O-phosphotransferases within the EPS matrix of *P. aeruginosa* biofilm bind to aminoglycosides and covalently modify specific amino or hydroxyl functions which make them inactive (Mingeot-Leclercq *et al.*, 1999). It has also been found that synergy between retarded diffusion and enzymatic destruction of antibiotics leads to resistance of *P. aeruginosa* biofilm (Mah and O'Toole, 2001). As the concentration of the antibiotic entering the cell is restricted, *P. aeruginosa* expresses beta-lactamases that destroy incoming antibiotics. Another resistance mechanism called efflux pump located in the cytoplasmic membrane is expressed during antibiotic treatment with azithromycin, gentamicin, tobramycin and ciprofloxacin through the regulation of efflux pump genes (Zhang and Mah, 2008). In *Escherichia coli* (*E. coli*), the transenvelope AcrAB-TolC MDR pump and chloramphenicol pump (CmIA) synergistically pick up chloramphenicol transported into the periplasm and extrude them out of the cell (Lee *et al.*, 2000).



**Figure 1.4** The delivery of an antibiotic and target binding. Under normal conditions, a pro-antibiotic is delivered to a cell, an enzyme converts it into a reactive form that locates and binds the target resulting in cell death (Lewis, 2007).

### 1.3.1 Persister cells in Biofilms

The inability of potent antibiotics even at high concentrations to completely inhibit or eradicate biofilms is possibly due to the presence of persister cells (Lewis, 2007). Persister cells have been described as metabolically inactive cells that account for approximately 1% of the total biofilm population (Anderson and O'Toole, 2008; Hall–Stoodley and Stoodley, 2009; Lewis, 2001; Lewis, 2007). It has been suggested that, these metabolically inactive cells are tolerant to antibiotics even at concentrations 10 to 100–fold higher than their minimum inhibitory concentrations and are responsible for relapse of biofilm infections through dispersal and colonisation of new niches (Lewis, 2001; Lewis, 2007). Though the mechanisms underlying the formation of persister cells remain an unknown, it has been shown that the highest rate of persister formation is at stationary phase of growth and independent of quorum sensing as spent growth media and early exponential cultures of *E. coli* or *P. aeruginosa* added together did not show appreciable increase in the number of persister cells isolated (Lewis, 2007). As they are metabolically inactive, persister cells do not express target sites for antibiotic activity. They are also known to express toxin–antitoxin systems where toxin molecules block antibiotic target sites (Lewis, 2005). Unlike resistant cells, persister cells do not need to undergo genetic modification to escape the effects of antibiotic and do not undergo genetic change (Wood *et al.*, 2013).

It has also been shown that translation takes place in persister cells at a very low rate (Gefen *et al.*, 2009; Shad *et al.*, 2006). However, aminoglycosides such as gentamicin that target ribosomes have weak activity towards persister cells. Allison *et al.* (2011) demonstrated that in metabolically–stimulated persister cells (from both Gram–negative and Gram–positive bacteria), aminoglycosides like gentamicin rapidly induce the killing of the cells at a greater rate when augmented with specific metabolites such as glucose, mannitol, fructose and pyruvate. When this was repeated in the presence of other antibiotics such as the quinolones and  $\beta$ –lactams, there was no significant change in killing of persister cells. They proposed that quinolones and  $\beta$ –lactams augmented with metabolic stimuli do not convert persister cells to an active state where DNA and cell wall synthesis take place.

### 1.3.2 Bacterial cell–cell interaction during biofilm formation

In biofilms, bacteria attach to each other, to host cells or the extracellular matrix by means of their surface proteins and sugars. Cell–surface proteoglycans, glycoproteins and glycolipids mostly contain oligosaccharide residues that are specific to cell types. Cell–surface appendages such as fimbriae expressed by *E. coli* and *P. aeruginosa* have been found to provide structural stability during biofilm formation (Lopez *et al.*, 2010). For example, type I fimbriae expressed by *E. coli* bind to mannose–containing receptors during biofilm formation. Bacterial lectins are carbohydrate–binding proteins that specifically identify and bind to sugar moieties to facilitate cell–cell or cell–matrix attachments during biofilm formation. This makes lectins useful primary diagnostic tools for the identification of bacterial species or cellular components (Afrough *et al.*, 2007; Munoz *et al.*, 1999; Munoz *et al.*, 2003; Slifkin and Doyle, 1990). *P. aeruginosa* has been found to express two important lectins, LecA and LecB that bind specifically to D–galactose and L–fucose respectively during biofilm development (Diggle *et al.*, 2006; Tielker *et al.*, 2005). The production of LecA and LecB has been found to be directly regulated by quorum sensing genes (Winzer *et al.*, 2000). Using immunoblot analysis, it was observed that quorum sensing gene *rhl* locus was directly responsible for the production of both LecA and LecB. This was confirmed when both lectins were lost in a mutant strain but restored after the introduction of a plasmid–borne *rhl* locus. However, in *lasR* mutant strains the commencement of lectin synthesis was found to be delayed (for about 18 hours) but not completely abolished. This is a demonstration that quorum sensing regulation of lectin synthesis is an essential mechanism for bacterial interaction during *P. aeruginosa* biofilm development. Therefore, the determination of quorum sensing genes associated with biofilms can be a useful diagnostic tool in differentiating between bacterial strains as either biofilm producers or non–biofilm producers.

### 1.4 Quorum sensing in bacterial biofilms

In a biofilm communication between bacterial cells (inter and intraspecies) is made possible by the production, secretion and recognition of certain chemical signals called quorum sensing (QS) molecules in the extracellular matrix. At a significant threshold (quorum), the concentration of these diffusible signalling molecules corresponds to a particular or confined



cell population density in the extracellular matrix. This recognition of cell population density–dependent signalling cascade induces a coordinated change in the gene expression profiles of the communicating bacteria in a process called quorum sensing (Cooper, 2010; Hammer and Bassler, 2003; Suga and Smith, 2003).

Quorum sensing molecules are the signalling molecules responsible for communication among cells growing on a surface or in a biofilm. They are also called autoinducers (Hammer and Bassler, 2003; Suga and Smith, 2003). Autoinducers regulate the expression of genes in bacteria and extensively control their responses (Cooper, 2010; Hammer and Bassler, 2003; Shirtliff *et al.*, 2002; Suga and Smith, 2003). Some of the responses that are controlled by quorum sensing circuits include motility, virulence, spore formation, biofilm formation, antibiotic resistance, conjugation, competence, pigmentation and bioluminescence (Hammer and Bassler, 2003; Nealson *et al.*, 1970). Hammer and Bassler (2003) emphasised that some bacteria in a consortium have the ability to detect and respond to multiple autoinducers allowing them to differentiate between the various species thereby harmonizing behaviours common to their community. This peculiar characteristic enables them to undertake certain activities as a multicellular group other than planktonic cells.

#### **1.4.1 Quorum Sensing Molecules**

The first evidence of quorum sensing was reported in the marine luminescent bacterium *Vibrio fischeri*, which can exist either as free–living or in an association with their fish or squid (*Euprymna scolopes*) symbiotic hosts (Nealson *et al.*, 1970). As free–living cells and at low cell densities, they do not express the luciferase–encoding genes which control light emission. However, when living in a specialised light organ of their symbiotic host, they express these genes as their cell density increases in relation to a correspondent increase in the production of signal molecules (Nealson *et al.*, 1970). At a significant threshold, the signal molecules activate bioluminescence (Nealson *et al.*, 1970). In a separate study by Eberhard *et al.* (1981), the autoinducers responsible for bioluminescence in *V. fischeri* was found to be 3–Oxo–C6–homoserine lactone, a member of the family of N–acyl–homoserine lactones.

Two types of autoinducers have been reported in bacterial biofilms. For example, the marine bacterium *Vibrio harveyi* produces and responds to signals from acylated homoserine lactone

(AHLs) called HAI-1 synthesised by LuxLM and the new furanosyl borate diester autoinducer known as AI-2 synthesised by LuxS (Chen *et al.*, 2002; Hammer and Bassler, 2003; Schauder *et al.*, 2001).

AHLs have been reported and proposed to mediate intraspecies communication solely between Gram-negative bacteria (Williams, 2007). Though different forms of AHLs are produced by different species; all the different AHLs have a common homoserine lactone ring moiety with varying length, degree of saturation and specific substitutions within an attached acyl side-chain (Rickard *et al.*, 2010). The AI-2 molecules are a family of inter-convertible molecules derived from the same precursor molecule; 4,5-dihydroxy-2,3-pentanedione (DPD) that can be detected by over 40 species of both Gram-negative and Gram-positive bacteria (Semmelhack *et al.*, 2005; Sun *et al.*, 2004; Surette and Bassler, 1998; Surette *et al.*, 1999, Xavier and Bassler, 2003). AI-2 has been shown to mediate signal transduction between intra-species and inter-species bacteria in the formation of biofilm and virulence in *Vibrio cholera* and *Streptococcus pneumoniae* and bioluminescence in *Vibrio harveyi* (Bassler and Losick, 2006; Rickard *et al.*, 2006; Schauder *et al.*, 2001; Surette *et al.*, 1999; Yoshida *et al.*, 2005).

#### 1.4.2 Signal Transduction in Gram-Negative Bacteria

Different quorum sensing communication circuits exist in both Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, the main QS system comprises of a LuxI synthase homolog, LuxR receptor homolog and acyl-homoserine lactones signalling molecules (Brackman *et al.*, 2011). The formation of cytoplasmic acyl-homoserine lactone is catalysed by the LuxI proteins of *V. fischeri* and its homologs, from the metabolic products S-adenosyl methionine and a suitable acyl-acyl carrier protein from the fatty acid biosynthetic pathway (Val and Cronan, 1998). Over 50 different bacterial species produce AHLs with each associated with a specific type of LuxI protein. Acylated homoserine lactones mediate signal transduction in Gram-negative bacteria. AHLs found in Gram-negative bacteria include *V. fischeri* (3-Oxo-C6-homoserine lactone (HSL)), *Pseudomonas aeruginosa* N-3-Oxo-C8-HSL), *Serratia liquefaciens* (-C4-HSL), and *Agrobacterium tumefaciens* (3-Oxo-C8-HSL) (Suga and Smith, 2003). In *P. aeruginosa*, two AHL quorum sensing systems are found; *las* and *rhl*. Each system is made up of its own AHL synthase

designated as LasI and RhII, and corresponding transcriptional regulator also designated as LasR and RhIR (Lopez *et al.*, 2010). In *P. aeruginosa*, AHLs in the cytoplasm bind to DNA-binding regulatory proteins, LasR, produced by *lasR* regulatory genes. The LasR-AHLs complexes subsequently recognise and bind to a number of genes and promoters under quorum sensing regulation activating the transcription of these genes in eliciting specific quorum sensing response (Shirtliff *et al.*, 2002; Suga and Smith, 2003).

### 1.4.3 Signal Transduction in Gram-Positive Bacteria

Quorum sensing in Gram-positive bacteria is mediated by a two-component signal transduction mechanism; a histidine kinase and a response regulator domain that elicits signal transduction through phosphorylation. The quorum sensing molecules are called auto-inducing peptides which are produced either after post-translational modification or unmodified oligopeptides. The ATP-binding cassette exporter protein then releases the mature oligopeptide into the cytoplasm where they are detected by the two-component signal transduction system. The histidine portion of the oligopeptide is autophosphorylated by the sensor kinase. The phosphate is subsequently transferred onto an aspartate residue found on the response regulator domain or protein. The complex formed then activates the expression of targeted genes under quorum sensing control (Demain, 1998). An example of a modified autoinducing peptide that is released by *Staphylococcus aureus* is the cyclic thiolactone, the accessory gene regulator quorum sensing system (AgrD) (Bassler and Losick, 2006).

### 1.4.4 Interspecies signal transduction and bioluminescence in *Vibrio* species

It has been demonstrated that, the bioluminescent marine bacterium *V. harveyi*, is capable of regulating some cellular processes such as bioluminescence, type III secretion, siderophore, polysaccharide and metalloprotease production using 2 parallel QS systems (Bassler *et al.*, 1993; Bassler *et al.*, 1994; Henke and Bassler, 2004; Lilley and Bassler, 2000; Nealson and Hastings, 1979). In the first system, *N*-(3-hydroxybutanoyl) homoserine lactone (HSL) also denoted as HAI-1 for *V. harveyi* autoinducer is produced and detected by LuxM and LuxN respectively (Bassler *et al.*, 1993). The second system produces AI-2 (3A-methyl-5,6-dihydro-furo(2,3-D) (1,3,2) dioxaborole-2,2,6,6A-tetraol), by LuxS which is detected by LuxP and LuxQ. The 2 QS systems which have been described in details in sections 5.2.2 and 5.2.3 in Chapter 5 are known to synergistically regulate multiple genes in addition to the

*luxCDABE* operon, responsible for light production in *V. harveyi* (Miller and Bassler, 2001). In an *E. coli* – *V. harveyi* mixed culture, exogenous AI-2 produced by *E. coli* was found to induce bioluminescence in *V. harveyi* (Xavier and Bassler, 2005a).

#### 1.4.5 Detection of QS signalling molecule production in bacteria

In addition to the PCR amplification of QS-associated genes, biosensor reporter systems have been used to detect short, medium and sometimes long acyl chains of AHLs (Steindler and Venturi, 2007). For example, the antibacterial purple pigment violacein produced by *Chromobacterium violaceum* is regulated by the CviI/R QS system which secretes and responds to both short and medium acyl chains of AHLs including C4-AHL and C8-3-oxo-AHL (McClellan *et al.*, 1997). In order to detect the production of AHLs with long acyl chains, the SinI/R system of *Sinorhizobium meliloti* (previously called *Rhizobium meliloti*) was constructed (Llamas *et al.*, 2004). This reporter system with a *sinI-lacZ* transcriptional fusion, incorporated a radiotracer, (normally <sup>14</sup>C label from (<sup>14</sup>C) methionine into AHLs) and could produce and respond to long acyl chains of AHLs including C12-AHL, C14-3-oxo-AHL, C16-AHL and C18-AHL (Llamas *et al.*, 2004). Some biosensors have also been constructed to detect AHLs at the single-cell level (Riedel *et al.*, 2001). An example of this is the QS system in *Burkholderia cepacia* with a *cepI-gfp* (green fluorescent protein) transcriptional fusion that enables the detection of AHLs using epifluorescence microscopy.

The creation of a broad host range reporter plasmids (Jiwaji, 2006; Jiwaji *et al.*, 2008; Jiwaji and Dorrington, 2009; Mather *et al.*, 2013) and the use of small molecule probes (Lowery *et al.*, 2008; Lowery *et al.*, 2013) have made it possible to explore and clarify some complex signalling pathways underlying molecular and cellular level behaviours of Gram-negative bacteria. In one of such studies, Lowery *et al.* (2013) introduced an AI-2 inhibitor (propyl-4,5-dihydroxy-2,3-pentanedione) into Lrs QS system fused to a *lacZ* reporter gene to study the proteome of *Salmonella enterica* serovar *typhimurium*. They discovered that QS inhibitors developed in a reporter strain also had effect on wild-type strains. For example, some proteins belonging to the Lsr family of proteins (LsrK, LsrB, LsrF, LsrA) in the well-characterised *lsr*-regulated QS system were selectively downregulated in both biosensor reporter and wild-type strains. However, AI-2 production by the LuxS QS system and associated proteins (AI-2 synthase and Pfs) in both strains were not affected.

In another study, Matcher *et al.* (2013) suggested among other reasons that the regulation of pyrimidine metabolism in *Pseudomonas putida* RU–KM3<sub>s</sub> could be through quorum sensing which did not require a well–characterised *lux* QS system. They used a *gus* ( $\beta$ –glucuronidase) reporter gene fused to dihydropyrimidinase (*dhp*) transcriptional promoter gene in the presence of the substrate (hydantoin) to monitor pyrimidine metabolism in *P. putida* RU–KM3<sub>s</sub>. The significance of the above–mentioned studies is that biosensor reporter strains make it possible to detect a wide range of AHLs.

#### 1.4.6 Quorum sensing inhibition

The contribution of quorum sensing molecules to enhance virulence and development of biofilms has promulgated the idea that the inhibition of quorum sensing might also contribute to the controlling of biofilms (Cooper, 2010). For example, the role of natural products such as garlic has been indicated in some studies to inhibit quorum sensing in *P. aeruginosa* (McLean *et al.*, 2004). Bjarnsholt *et al.* (2005) have also indicated the use of garlic in treating bacterial infections as it was effective against *P. aeruginosa* isolated from cystic fibrosis patients. In their work, Bjarnsholt *et al.* (2005) observed that, garlic–treated *P. aeruginosa* biofilms grown in an *in vitro* continuous culture once–through flow chambers were susceptible to tobramycin and phagocytosis by polymorphonuclear leucocytes. Garlic extract has previously been reported to have antimicrobial properties and by using DNA microarray–based transcriptomic analysis has also been found to inhibit 167 gene expressions in *P. aeruginosa*, 92 of which are quorum sensing–regulated (Ankri and Mirelman, 1999; Rasmussen *et al.*, 2005).

The theory of quorum quenching; the use of enzymes and inhibitors such as antagonists of autoinducers to block and disrupt quorum sensing systems thereby preventing their responses; has been well received as a means of controlling biofilms (Suga and Smith, 2003; Zhang and Dong, 2005). AHL–degrading enzymes have been identified from some prokaryotes and eukaryotes based on studies from the characterisation of acylated homoserine lactones. Some of these degrading enzymes and their sources include AHL lactonase–*Bacillus sp.*240B1 and *Klebsiella pneumonia*, AHL acylase–*Variovorax paradoxus* VAI–C, acylase I – porcine (kidney) and lactone–human airway epithelia (Dong *et al.*, 2000; Greenberg *et al.*, 2004; Leadbetter and Greenberg 2000; Xu *et al.*, 2003).

Modifications of natural AHLs or AIs have been found to be potent antagonists. These include halogenated furanones produced from marine algae *Delisea pulchra* which interfere with AHL-mediated quorum sensing which affect *P. aeruginosa* biofilm architecture and subsequent bacterial detachment (Hentzer *et al.*, 2002). Again these natural products are able to interfere with the SwrR-C4-HSL interaction in *Serratia liquefaciens* and also inhibit the LuxR-3-Oxo-C6-HSL interactions in *V. fischeri*, *Erwinia carotovora*, *Erwinia chrysanthemi* and *Yersenia enterocolitica* (Hentzer *et al.*, 2002).

It is highly likely that the continued search for more quorum sensing inhibitors and enzymes will result in the next ground-breaking success in the treatment of biofilm-infected chronic wounds such as diabetic foot ulcers.

## 1.5 Thesis hypothesis and aims

Quorum sensing and glycan-lectin interactions are two mechanisms in biofilm development that have provided important information in understanding the role of bacteria in chronic infections. The inhibition of quorum sensing as well as glycan-lectin interactions have been considered as alternative strategies to antibiotic treatment that can be useful in the treatment of chronic infections through the prevention and disruption of biofilm formation. Quorum sensing inhibitors (QSI) such as cinnamaldehyde (CA) baicalin hydrate (BH) and 2(5H)-furanone have been found to influence matrix production and biofilm formation but do not stop bacterial growth in their planktonic state (Brackman *et al.*, 2011). QSIs were found to increase the susceptibility *P. aeruginosa* and *S. aureus* biofilms to conventional antibiotics such as tobramycin, clindamycin and vancomycin, leading to increased reduction in biofilm.

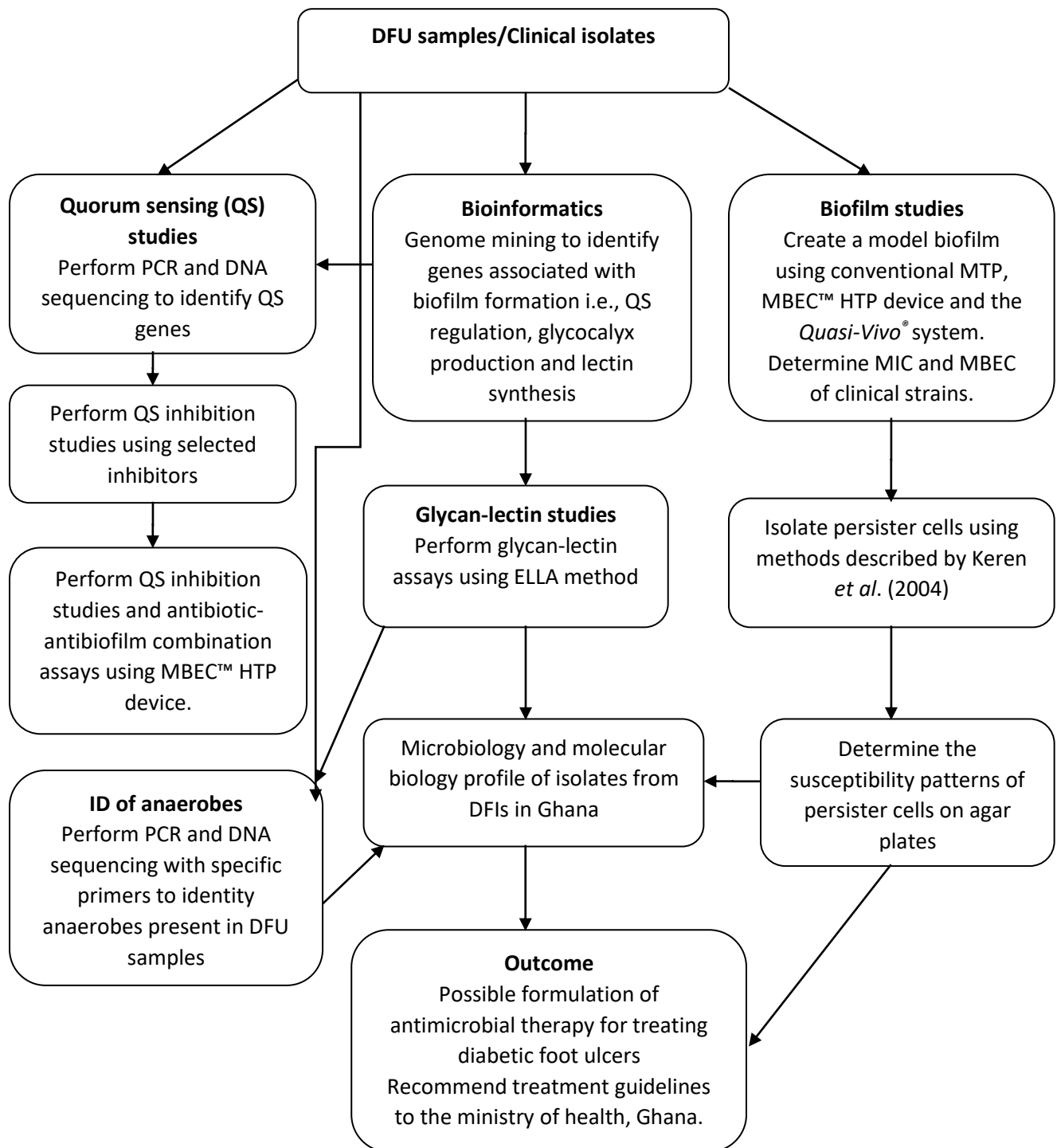
The use of lectin-inhibiting carbohydrates such as  $\alpha$ -methyl-galactoside and  $\alpha$ -methyl-fucoside which specifically inhibit LecA and LecB lectins expressed by *P. aeruginosa*, have been found to reduce adhesion, lung injury and cell mortality when grown *in vitro* with A549 cells and *in vivo* in a murine model (Chemani *et al.*, 2009). Zinc has recently been found to have antibiofilm activity in inhibiting biofilm formation in both Gram-negative and Gram-positive pathogens (Wu *et al.*, 2013). However, the mechanism by which this takes place has not been characterised.

The hypothesis for this work was that the chronic nature of diabetic foot ulcers is maintained by bacterial communication and cellular interaction. The aim of the present study was to characterise the microbial profiles of diabetic foot ulcers and investigate their role in maintaining the chronicity of the wound. The development of antimicrobial combination strategies to effectively disrupt and/or eradicate biofilm by inhibiting quorum sensing and molecular interactions that underline biofilm formation was also investigated. The following objectives will be investigated in an attempt to achieve the aims of this study;

- To characterise, determine the prevalence rate, and identify the causative agents of diabetic foot infections among the diabetic population attending the Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana.
- To determine the antibiotic susceptibility patterns of DFU isolates and study their genetic relatedness using bioinformatics tools.
- To investigate the existence of biofilms in DFIs through the model creation of a biofilm using conventional 96-well microtitre plates, MBEC™ HTP assay and the *Quasi-Vivo*® system.
- Detect quorum sensing mechanisms during biofilm formation.
- Perform glycan-lectin analysis to study bacterial interactions during biofilm formation.
- To study the effects of the combinations of antibiotics and other antibiofilm agents in the eradication of biofilms using the high-throughput MBEC™ assay.

### 1.5.1 Research Plan

Throughout the period of this study the flow chart below served as a guide in achieving the aims and objectives of the study with illustrations on how the experimental work will be carried out.





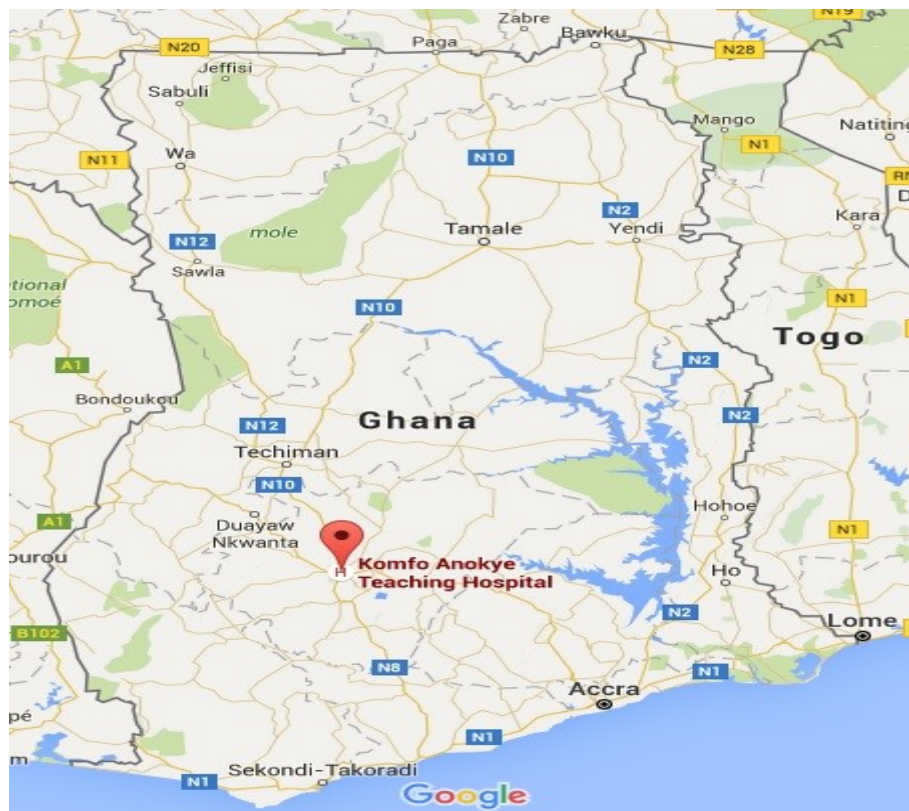
## CHAPTER 2

# Characterisation of diabetic foot isolates (Materials and Methods)

## 2.1 Sample collection and processing

### 2.1.1 Study location

This study was a collaborative work between the University of Westminster, London and the Komfo Anokye Teaching Hospital (KATH) Kumasi, Ghana. KATH was chosen as the sample collection site for three reasons; it is the second largest referral and teaching hospital in Ghana located close to the centre of the country and receives a lot of referral cases mostly from the northern part of Ghana and other surrounding regions; it is the most populated region in Ghana (according to the 2010 Population and housing Census) with a population of 4.8 million representing 19.4% of the total population (24.6 million); and one-third (34.3%) of its residents have migrated to the region. Therefore, the location of KATH in the Ashanti region was perfect for the purpose of the current study as the Ashanti region provides a representative population for Ghana as far as the 2010 national census demographics are concerned.



**Figure 2.1** Map of Ghana showing the location of the Komfo Anokye Teaching Hospital, (KATH) in Kumasi, Ashanti Region. (Image source: Google Maps)

### **2.1.2 Ethical approval**

Ethical approval for participants' recruitment and sample collection for this study was granted by the Research and Development Unit at KATH, Kumasi, Ghana (Appendix E3). The study was also registered and approved by the Research and Development Unit at KATH (Appendix E4). Ethical considerations for this study were further reviewed and approved by the University of Westminster Research Ethics Sub Committee, London (Appendix E1). In addition, facilities in the Clinical Microbiology department at KATH were permitted for reception, processing and storage of clinical samples throughout the sample collection period in Ghana.

### **2.1.3 Participants' recruitment**

Participants in this study were diabetic foot ulcer patients attending the Diabetes Centre at KATH, Kumasi. They included both old and new patients attending the centre. Participants were voluntarily recruited for the study and provided with Participant Information Sheet (PIS, Appendix E5) which contained thorough information about the project as well as their participation in the project. Recruited participants were asked to voluntarily sign consent forms (Appendix E5) copies of which were retained by the Diabetes Centre, KATH.

### **2.1.4 Wound sampling and classification of DFUs**

Between January 2011 and December 2014, 356 wound samples were taken from diabetic foot ulcers (DFUs) who attended the Diabetes Centre, KATH. They included both old and new patients. Before the ulcers were sampled, the skin around the edges of the wound was first disinfected with 70% (v/v) ethanol and the surface area of the wound moistened with sterile water or saline. Wound sampling was performed by introducing a sterile cotton-tipped swab into the ulcer of the affected patient and swabbing with the cotton-tipped end to collect exudates that were likely to contain bacteria for further processing. In order to recover more bacteria from the ulcer, the wound was carefully swabbed by rotating the cotton-tipped end across the surface area in a zig-zag motion. All patients were sampled only once. Wound samples collected included swabs and pus from foot ulcers only. Swabs were also taken from recurrent wounds on amputated feet. All other wounds apart from foot ulcers were excluded. They included hand wounds, decubitus ulcers and leg ulcers. All wound samples were

cultured within 2 hours of collection. No sample was refrigerated overnight before culture. Clinical isolates recovered from these samples were subsequently employed in all investigations throughout the duration of this project.

Thirty-eight of the 356 DFU samples collected between February and April, 2013 were classified according to Wagner's ulcer classification grade which is based on the depth of penetration, the presence/absence of gangrene and the degree of tissue necrosis (Wagner, 1987). Initial processing of all wound samples was performed at the microbiology department of Komfo Anokye Teaching Hospital (KATH), Kumasi. Further processing of all samples was done at the Molecular Biology/Microbiology laboratory, Cavendish Campus, University of Westminster, London

## **2.2 Materials**

Materials used in this study were purchased from some selected companies including Sigma–Aldrich (Dorset, UK), VWR Ltd (East Grinstead, UK), Qiagen (Crawley, UK), and Fisher Scientific Ltd (Loughborough, UK). All biofilm assays were performed using both conventional microtitre plates, the MBEC™ device (MBEC™ HTP and P & G, Innovotech Inc, Edmonton, Alberta, Canada) and Kirkstall *Quasi Vivo*® system (Kirkstall Ltd, Rotherham, UK). All PCR and DNA sequencing kits were obtained from Qiagen and Sigma–Aldrich Ltd, UK. Solid media used for culturing bacterial strains were obtained from Sigma–Aldrich (Dorset, UK), VWR Ltd (East Grinstead, UK), Oxoid Ltd (Basingstoke, UK) and Fisher Scientific Ltd (Loughborough, UK). They included Luria–Bertani (LB) agar, MacConkey agar (MA), chocolate agar (CA), blood agar (BA) and Kolliphor 407® gel.

### **2.2.1 Bacterial strains**

#### **2.2.1.1 Reference/control strains used in this study**

A list of control strains used in the present study is provided in Table 2.1. *Vibrio harveyi* NCIMB 1280 is a luminous strain that uses the *luxCDABEGH* operon to produce light through quorum sensing whereas *V. harveyi* NCIMB 1872 is the aldehyde mutant of the former and cannot produce light even in the presence of externally supplied autoinducer and aldehyde substrate (Bassler *et al.*, 1993; Meighen, 1991; Neilson and Markovitz, 1970).

Control strains, *E. coli* NCTC 10418, *P. aeruginosa* PAO1 and *S. aureus* NCTC 6571 have previously been demonstrated as biofilm producers (Lopez *et al.*, 2010).

**Table 2–1** Reference/control strains used in this study

Bacterial Strains*	Description	Purpose
<i>Vibrio harveyi</i> NCIMB 1280	AI–2 mutant (LuxM <sup>–</sup> LuxS <sup>–</sup> LuxCDABE <sup>+</sup> )	Detection of exogenous AI–2 quorum sensing molecules for bioluminescence
<i>Vibrio harveyi</i> NCIMB 1872	Aldehyde mutant, (LuxS <sup>–</sup> , LuxLMN <sup>–</sup> LuxCDE <sup>–</sup> )	Negative control for <i>luxCDE</i> and bioluminescence activities
<i>Agrobacterium tumefaciens</i> NCIMB 14543	pMJ258, LacZ <sup>+</sup>	Broad range host – For detection of AI–1 quorum sensing molecules in Gram–negative bacteria.
<i>Escherichia. coli</i> NCTC 10418	Fully susceptible	Antibiotic susceptibility testing and biofilm formation
<i>Pseudomonas aeruginosa</i> PAO1	LasI <sup>+</sup> , RhIR <sup>+</sup>	Quorum sensing and biofilm assays.
<i>Staphylococcus aureus</i> NCTC 6571	Fully susceptible	Antibiotic susceptibility testing and biofilm assay

\*– Bacterial strain: *V. harveyi* reference strains NCIMB 1280 and 1782 including *A. tumefaciens* NCIMB 14543 were purchased from the National Collection of Industrial and Marine Bacteria (NCIMB) Ltd, Aberdeen, Scotland, *E. coli* NCTC 10418, *P. aeruginosa* PAO1 and *S. aureus* NCTC 6571 were obtained from Dr Patrick Kimmitt’s stock, University of Westminster, London.

### 2.2.1.2 Identification, maintenance and growth conditions DFU isolates

DFU samples including 38 samples collected between February and April, 2013 were directly cultured on freshly prepared nutrient (NA), Blood, Chocolate and MacConkey agar and incubated overnight 37°C under aerobic conditions. In addition, all 38 DFU samples were cultured on fastidious anaerobic agar (FAA) and incubated overnight at 37°C under anaerobic conditions. FAA cultures were incubated for up to 48 hours using anaerogen (Oxoid, Basingstoke, UK) and gas generators. Gram staining was performed on all isolated bacteria.

Pure colonies from overnight cultures were selected for biochemical identification using API10S and RapiD 20E kits (bioMérieux, France). All tests were carried out according to the manufacturer's instructions. In all 407 bacterial strains were isolated from 356 DFU samples throughout the period of this study. Fifty of these strains were subsequently used as representative strains for further analyses.

Positive cultures were subcultured on LB agar and in 10 mL LB broth, incubated overnight and processed for storage. All bacterial cultures were maintained at  $-80^{\circ}\text{C}$  in LB broth supplemented with 50% (v/v) glycerol. Sea water broth was used for maintaining *Vibrio harveyi* strains NCIMB 1280 and NCIMB 1872. Prior to their use, *V. harveyi* strains NCIMB 1280 and NCIMB 1872 were cultured on L-marine agar or sea water agar (Appendix A1) and incubated at  $25^{\circ}\text{C}$  and  $20^{\circ}\text{C}$  respectively for up to 48 hours under aerobic conditions. *Agrobacterium tumefaciens* strain NCIMB 14543 was cultured on LB agar or in 10 mL LB broth supplemented with  $0.05\mu\text{g/mL}$  Kanamycin and incubated overnight at  $30^{\circ}\text{C}$  under aerobic conditions before each assay. All other bacterial strains were cultured on LB agar or in LB broth unless otherwise stated.

## **2.2.2 Preparation of media, buffers and stock solutions**

### **2.2.2.1 Solid media**

Solid media used in this study were prepared according to manufacturer's instructions. They included LB agar, L-marine (LM) agar, MacConkey agar, Chocolate agar, sea water agar and Kolliphor 407<sup>®</sup> gel. Briefly, the desired amounts of the powdered agar were weighed and dissolved in deionised water and thoroughly mixed either by heating on a hot plate or gently stirring and swirling till all the powdered agar is dissolved. The dissolved contents were then autoclaved at  $121^{\circ}\text{C}$  for 15 minutes. In the preparation of solid media supplemented with blood, antibiotics or sugars, the autoclaved media were left to cool down to about  $50^{\circ}\text{C}$  after which the supplements were added. Blood agar was normally prepared as 10% (v/v) of LB agar.

### **2.2.2.2 Liquid media and buffers**

All liquid media were prepared according to manufacturer's instruction. All liquid media were sterilised by autoclaving at  $121^{\circ}\text{C}$  for 15 minutes before use. Liquid media used in this

study included LB broth, LM medium for *V. harveyi* growth and bioluminescence assays, sodium thioglycolate bacterial recovery medium, trypticase soy broth (TSB), BM medium, AB medium.

Buffers used in this study were prepared according to manufacturer's instructions and sterilised by autoclaving. They included phosphate buffered saline (PBS), coaggregation buffer, 50X Tris–Acetate–Ethylene–diamine–tetra–acetic acid (TAE) buffer and TE (10 mM Tris–HCL, 1 mM EDTA, pH 8.0) buffer. Other solutions used in this study were, 0.9% physiological saline, 2.5% and 4% formaldehyde, 10 mM cetylpyridinium chloride, 10% (v/v) Ziehl Carbol fuchsin, 1% (w/v) Congo red, 0.1% (w/v) from (stock) Calcofluor white stain, 0.5mg/L ethidium bromide and 20mg/mL (w/v) 5–bromo–4–chloro–3–indolyl– $\beta$ –D–galactopyranoside (X–gal).

### **2.2.2.3 Preparation of antibiotics and antimicrobials and quorum sensing inhibitors**

Antibiotics (powder form) and antimicrobials (either liquid or crystalline powder form) used in this study were prepared as stock solutions using their respective solvents and according manufacturer's instructions (Tables 2.2 and 2.3). They included ampicillin (AMP), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GM), levofloxacin (LEV) and kanamycin (KAN). Stock solutions for all antibiotics used in this assay were prepared according to the Clinical Laboratory Standards Institute (CLSI, document M100–S24) and British Society for Antimicrobial Chemotherapy (BSAC) methods for antimicrobial susceptibility testing guidelines, filter–sterilised and stored at  $-80^{\circ}\text{C}$  (British Society for Antimicrobial Chemotherapy, 2013; Clinical Laboratory Standards Institute, 2014). Working solutions of each antibiotic used for minimum inhibitory concentration (MIC) determinations were prepared as 10, 100 and 1000–fold concentrations using the MIC range (512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25  $\mu\text{g}/\text{mL}$ ) as a guide and stored at  $-20^{\circ}\text{C}$ . Working concentrations of each were prepared as a 5120  $\mu\text{g}/\text{mL}$ . The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used as guidelines for the interpretation of MIC data where BSAC and CLSI did not provide enough guidelines. The MIC breakpoints for ceftazidime and levofloxacin against members of the *Enterobacteriaceae* family were given as 0.125 – 4.0 mg/L, and 0.25 – 4.0 mg/L respectively (The European Committee on Antimicrobial Susceptibility Testing, 2015).

**Table 2–2** Stock solutions of some antibiotics and their concentrations

<b>Antibiotic</b>	<b>Solvent/diluent</b>	<b>Concentration (mg/mL)</b>
Ampicillin	Water	50
Ceftazidime	Sodium Carbonate/Water	50
Ciprofloxacin	Water	50
Gentamicin	Water	50
Levofloxacin	1:1 Water/0.1 M NaOH	100
Kanamycin	Water	25

Antimicrobials and quorum sensing inhibitors (QSIs) used in the present study included, polymyxin B (PMB) and polymyxin B nonapeptide (PMBN), (Z)-4-Bromo-5-bromomethylene-2(5H)-furanone (2(5H)-furanone), baicalin hydrate (BH) and cinnamaldehyde (CIN). The above mentioned QSIs and the two antimicrobial peptides were purchased from Sigma Aldrich (Dorset, UK). Stock solutions of the QSIs and the antimicrobial peptides were prepared according to manufacturer’s instructions and stored at –20°C (Brackman *et al.*, 2011; Conrad, 1995). Working solutions of both QSIs and antimicrobial peptides were stored at 2 – 8°C for not more than 2 weeks.

**Table 2–3** Antimicrobials/QSIs and their working concentration

<b>Antimicrobial/QSI</b>	<b>Solvent/diluent</b>	<b>Concentration (µg/mL or µM)</b>
Baicalin hydrate	Water	25 <sup>a</sup>
Cinnamaldehyde	Ethanol/methanol	1000 <sup>b</sup>
2(5H)-Furanone	Water	500
PMB	Water	500
PMBN	Water	500

<sup>a</sup> – units for baicalin hydrate in mg/mL; <sup>b</sup> – units of cinnamaldehyde in µM (Brackman *et al.*, 2008; Brackman *et al.*, 2011; Brackman and Coenye, 2015; Clinical and Laboratory Standards Institute, 2007).



#### **2.2.2.4 Neutraliser recovery media for MBEC™ assay**

In order to accurately determine the bactericidal concentrations of the antibiotics used in the MBEC™ assay, the neutraliser recovery media (NRM) recommended by the manufacturer was used. NRM is a 2.5% (v/v) solution made up of 500 µL universal neutraliser solution and a 20 mL recovery media. The purpose of the universal neutraliser solution is to reduce toxicity of biologically active agents that are carried over from the antimicrobial challenge plate to the recovery media. The universal neutraliser was made up of L-histidine, L-cysteine and reduced glutathione dissolved in sterile distilled water and sterilised with a 0.22 µm filter (Merck Millipore Ltd, Watford, UK). This solution was stored at –20°C.

The recovery media was prepared with Mueller–Hinton broth supplemented with saponin and Tween–80 (20 g/L and 10 g/L respectively) and sterilised by autoclaving. To recover planktonic cells after the antimicrobial challenge, the MBEC™ peg lid was transferred onto a 96–well plate containing the neutraliser recovery media.

Recipe for the preparation of some of the media, buffers and solutions can be found in Appendix A1 to A6.

### **2.3 Methods**

#### **2.3.1 Antibiotic susceptibility determination of DFU isolates**

All 407 bacterial isolates were tested for their antibiotic susceptibility patterns according to the British Society for Antimicrobial Chemotherapy (BSAC, 2015) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) methods for antimicrobial susceptibility testing. Antibiotic susceptibility testing (AST) was performed on Iso–Sensitest agar (Oxoid, Basingstoke, UK). About 3 or 4 colonies were picked with a sterile bacteriological loop and transferred into Iso–Sensitest broth (Oxoid, Basingstoke, UK). The turbidity of the suspension was adjusted to 0.5 McFarland using sterile distilled water. A 1:100 dilution of the suspension was made and evenly streaked on Iso–Sensitest agar plates using sterile cotton swabs in order to produce confluent growth.

### 2.3.2 Disc diffusion test

All DFU isolates were tested against the following selection of antibiotics (Axiom® Laboratories, India); ampicillin (A, 10 µg), ampicillin/sulbactam (AS, 20 µg), cotrimoxazole (COT, 25 µg), cefotaxime (CF, 30 µg), tazobactam/piperacillin (TZP, 100/10 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TE, 30), ceftazidime (CAZ, 30 µg), ofloxacin (OF, 5 µg), gentamicin (GM, 10 µg), amikacin (AK, 30 µg), levofloxacin (LEV, 5 µg) and vancomycin (VAN 5 µg). The antibiotic discs were placed on Iso–Sensitest agar the inoculated plates and incubated with aeration at 35 – 37°C for 18–24 hours. Zones of inhibition (ZOI) observed after incubation was measured with rulers and interpreted according to BSAC and EUCAST guidelines.

### 2.3.3 Growth curve of clinical strains

Two clinical strains namely; *K. pneumoniae* and *P. mirabilis* were used as representative strains to study the growth patterns of clinical isolates from DFUs. section 3.6 of Chapter 3 further outlines the reasons why *K. pneumoniae* and *P. mirabilis* were chosen as representative strains for the current study. *E. coli* NCTC 10418 was used as a control. Glycerol stock of each strain was cultured on LB agar (Sigma Aldrich, UK) and incubated overnight under aerobic conditions at 37°C. A colony from the overnight cultures was inoculated in 5 mL LB broth and incubated at 37°C overnight in a shaking incubator (VWR, UK) at 250 rpm. The optical densities (OD) of the broth cultures were read at 600 nm (OD<sub>600</sub>) using a spectrophotometer (Jenway 6300, Bibby Scientific Ltd, UK). A 1:1000 dilution of the cultures was made in 50 mL fresh LB broth in 125 mL conical flasks. The flasks were then incubated with aeration at 37°C in a shaking incubator for a period of 24 hours. A sample was removed from each flask at designated time points (1, 2, 3, 4, 5, 12, 18 and 24 hours) and their OD<sub>600</sub> determined.

To determine the growth of *K. pneumoniae* and *P. mirabilis* under the conditions cultivated, their total colony–forming unit per millilitre (CFU/mL), a ten–fold dilution was performed using 0.9% normal saline. Ten microliters (10 µL) of each diluted sample was spotted on LB agar (on MacConkey agar in the case of *P. mirabilis*) and incubated overnight at 37°C. The colonies were counted and the total viable count was expressed as CFU/mL of each culture

plate. Cultures were performed in duplicates and their mean value determined for subsequent analysis.

### 2.3.4 Coaggregation assay

The ability of DFU isolates to initiate biofilm formation and adhere to each other (intra- and inter-generically) during biofilm formation was assessed. The ability of bacterial isolates to adhere to each other in suspension is an important step in selecting bacterial strains for *in vitro* biofilm assays. Eight DFU isolates were selected for the coaggregation assay (Table 2.4). Two independent standard coaggregation techniques (visual and spectrophotometric assays) previously described by McIntire *et al.* (1978), Cisar *et al.* (1979) and Hill *et al.* (2010) were used in this study with some modifications.

**Table 2–4** Representative isolates for coaggregation assay

<b>DFU isolate ID</b>	<b>Bacterial species</b>
003a	<i>Citrobacter koseri</i>
001	<i>Proteus mirabilis</i>
015	<i>Escherichia coli</i>
038	<i>Pseudomonas aeruginosa</i>
025a	<i>Providencia stuartii</i>
021b	<i>Klebsiella pneumoniae</i>
005a	<i>Proteus mirabilis</i>
028b	<i>Klebsiella pneumoniae</i>

Firstly, bacterial strains were grown overnight in 30 mL BM medium in a shaking incubator at 35–37°C. BM medium was prepared according to the recipe (Appendix A3) given by Hill *et al.*, (2010). All strains were grown to a stationary phase to OD<sub>600</sub> 1.8 – 2.0 and harvested by centrifugation at 8000 x g for 10 minutes. Cells harvested from overnight BM cultures were washed twice in 30 mL coaggregation buffer. Coaggregation buffer (Appendix A4) was prepared by making a 1 litre solution made up of 0.1 mM CaCl<sub>2</sub>, 0.1 M MgCl<sub>2</sub>, 0.15 M NaCl and 1 mM Tris–HCl dissolved in distilled water. The solution was sterilised by autoclaving at 121°C for 15 minutes. Washed cells were subsequently resuspended in fresh coaggregation

buffer to give OD<sub>600</sub> of 2.0. Coaggregation test was carried out in pairs by mixing equal volumes (500 μL) of each cell suspension in clear Eppendorf tubes. The mixture was vortexed for 2 minutes and left to stand 2 hours at room temperature, briefly vortexed again before scoring. The coaggregation of each mixture of cell suspensions was scored and recorded according to the visual scale developed by Cisar *et al.* (1979). The tubes were then incubated overnight at room temperature, mixed briefly the next day and then scored again after 24 hours. Negative controls were made of 500 μL each of each cell suspension plus 500 μL of coaggregation buffer. Coaggregation score for each test was determined according the visual scale described by Cisar *et al.* (1979). Autoaggregation (within species) and coaggregation (between two different species) were visually scored on a scale of 0 – 4 and reported as follows; 0 – no visible aggregates; 1+ – small uniform aggregates; 2+ – definite visible aggregates without settling in suspension; 3+ – large aggregates settling easily leaving a turbid supernatant fluid; 4+ – large aggregates with clear supernatant which settles immediately after vortex.

In the second quantitative assay, bacterial strains were grown in BM medium as previously mentioned. This assay was performed using the method described by Cisar *et al.* (1979) with some modifications. For example, overnight cultures were grown without atmospheric nitrogen (as all bacterial species were grown under aerobic conditions) and harvested at OD<sub>600</sub> and not at OD<sub>650</sub> (Lane *et al.*, 2009). Overnight cultures were harvested when OD<sub>600</sub> was 1.5–2.0 and then centrifuged at 8000 x g for 10 minutes. The harvested cells were washed twice using a coaggregation buffer of pH 8 and containing 0.025 M potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) and 0.025 M sodium chloride (NaCl). Equal volumes (500 μL) of cell suspensions of a coaggregation pair were mixed together in clear (10 X 75 mm) tubes, allowed to stand for 10 minutes and mixed again. The cell suspension mixtures were then incubated at room temperature for 2 hours and centrifuged at 10 x g for 2 minutes. The supernatants were then collected and pipetted into 96–well plates and their absorbance determined at OD<sub>600</sub> using a microtitre plate reader. Controls were made up of each bacterial strain plus coaggregation buffer. The absorbance of each coaggregation pair was expressed as a percentage according to the formula given (below) by Shen *et al.* (2005).

$$\text{Percentage Coaggregation} = \frac{(\text{OD}_{600}X_1 + \text{OD}_{600} X_2) - 2 \times \text{OD}_{600}(X_1+X_2)}{\text{OD}_{600}X_1 + \text{OD}_{600}X_2} \times 100$$

Where; ( $X_1$ ) is the first coaggregation pair; ( $X_2$ ) is the second coaggregation pair in the cell suspension. Percentage coaggregation of each coaggregation pair above 5% was regarded as positive results for quantitative autoaggregation or coaggregation.

## 2.4 Biofilm studies

Bacterial biofilm formation and development have been studied using a variety of systems. These systems are mainly categorised into static and continuous flow systems. Preference for any of these *in vitro* systems depends on a number of reasons. For example, biofilm formation using static systems are helpful in studying early events during biofilm development (Merritt *et al.*, 2011). Furthermore, static biofilm systems are quite simple to use and have high-throughput making them adaptable for studying biofilms under diverse conditions. On the other hand, flow systems are useful for studying mature biofilms where continuous supply of growth media and adjustments of other biofilm growth requirements such as oxygen, pH, nitrogen and carbon dioxide are required.

In this study, both static and continuous flow biofilm systems were employed to determine the efficacy of these systems to study formation, inhibition and eradication of biofilms. The 3 systems that were used in this study were the conventional 96-well microtitre plate assay, MBEC™ assay and the Kirkstall *Quasi Vivo*® system.

### 2.4.1 Conventional microtitre plate (MTP) biofilm assay

This technique has previously been described as suitable for growing biofilms in a wide range of bacteria (Merritt *et al.*, 2011; O'Toole *et al.*, 1999; O'Toole, 2011; Stepanovic *et al.*, 2001). However, some modifications were made in this study. In place of water used in the washing step, 1X phosphate buffered saline (PBS) was used instead and 33% (v/v) acetic acid was used as solubiliser. Also, unbound cells from the 24-hour old biofilm cultures grown in the 96 – well microtitre plates were carefully aspirated before fixing with 4% formaldehyde followed by staining. This was done in order to recover most of the biofilm formed for further analysis. Culture media used to support the growth of the bacterial cells were LB (for aerobic cultures), brain heart infusion (BHI) and trypticase soy broth (TSB, with or without 0.25% glucose). In this study, 9 bacterial species designated as: 003a *C. koseri*, 015 *E. coli*, 018b *K. variicola*, 021a *P. mirabilis*, 021b *K. pneumoniae*, 025a *P. stuartii*, 028b *K.*

*pneumoniae*, 005 *P. mirabilis* and 038 *P. aeruginosa* in addition to 2 control species; *E. coli* NCTC 10418 and *S. aureus* NCTC 6571. Briefly, a 1:100 dilution of fresh broth culture was made and pipetted into the wells of the 96-well microtitre plate which was aerobically incubated overnight at 37°C. Broth suspension containing unbound cells and overlaying the biofilm was aspirated and the biofilm fixed with 4% formaldehyde for 10 minutes. The wells were washed with 1X PBS and stained with 0.1% crystal violet (CV) for 10 minutes. The stained biofilm was washed again with 1X PBS, allowed to air-dry and solubilised with 33% (v/v) acetic acid. Absorbance was measured at 570 nm with a microtitre plate reader using 33% (v/v) acetic acid only as blank. This assay was used to study biofilm under different environmental conditions. All tests were done in duplicates and repeated at least three times to make sure the results were reproducible. *E. coli* NCTC 10418 was used as a control strain in all replicates (Branda *et al.*, 2005). Biofilm production was defined according to the classification described by Pye *et al.* (2013). Biofilm producers were defined as; weak producers if OD<sub>570</sub> was  $\geq 0.05$  but  $< 0.13$ , moderate producers if OD<sub>570</sub> was  $\geq 0.13$  but  $< 0.25$  and strong producers if OD<sub>570</sub> was  $\geq 0.25$ .

#### **2.4.1.1 pH assay**

The ability of the selected clinical isolates to form biofilm in either acidic or alkaline environments was tested in growth media set at specific pHs. Clinical isolates were inoculated in LB broths of pH 4, 7 and 10 and incubated aerobically at 37°C overnight. However, the nutrient strengths of growth media were not altered. Biofilm growth at pH 7 was used as a control to compare the extent of biofilm growth in normal, acidified and alkaline broth cultures. Residual biofilm produced by each bacterial species under the different pH conditions was estimated using the conventional MTP biofilm assay described in section 2.4.1 (above) and the results classified according to the definitions by Pye *et al.* (2013).

#### **2.4.1.2 Temperature Assay**

Clinical strains were also cultured in LB broth at some selected incubation temperatures to determine their ability to form biofilm in extreme environmental conditions. Three (3) incubation temperatures (26, 37 and 42°C) were selected to grow biofilms. The nutrient strength of growth media was unaltered. Incubation temperature at 37°C was used as a

control to compare the extent of biofilm growth among the selected temperatures. Residual biofilm produced by each bacterial species at different temperature was estimated using the conventional MTP biofilm assay described in section 2.4.1 and the results classified according to the definitions by Pye *et al.* (2013).

#### **2.4.1.3 Nutrient concentration assay**

This assay was performed to determine whether clinical strains can form biofilm in diluted concentrations of nutrients in the media used to support their growth. The strains were grown in reduced nutrient concentrations of LB broth and their ability to form biofilms was compared. Stock concentration (25g/L) of LB broth was diluted to 12.5g/L and 6.25g/L and used to grow biofilms. Residual biofilm produced by each bacterial isolate at different nutrient concentrations was estimated using the conventional MTP biofilm assay described in section 2.4.1 and the results classified according to the definitions by Pye *et al.* (2013).

All biofilm assays under the environmental (pH, temperature and nutrient concentration) conditions described above, were performed in 3 replicates and the results statistically determined using GraphPad Prism statistical software (GraphPad Software, Inc., California, USA).

#### **2.4.2 Biofilm inhibition and eradication assays**

The ability of antibiotics to inhibit and/or eliminate biofilms formed by Gram–negative pathogens isolated from DFUs was assessed. Inhibition and eradication assays were performed with a focus on 2 representative facultative anaerobic Gram–negative strains namely; *K. pneumoniae* and *P. mirabilis*. They were selected due to their multidrug resistant nature and as strong biofilm producers. Antibiotics were selected with respect to their functionality, AST patterns and relevance to this study. The representative strains were tested against ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin (Table 2.1) to determine the minimum antibiotic concentrations needed to inhibit or eradicate the biofilms they produce.

#### **2.4.2.1 Minimum inhibition concentration (MIC) determination**

The MIC test was performed to determine the lowest antibiotic concentration that inhibited the growth of planktonic cells and also inhibited diabetic foot isolates from forming biofilm after overnight incubation. Working solutions of antibiotics were prepared as 10 and 100–fold concentrations of the MIC range as mentioned in section. A 1:100 dilution (corresponding to 0.5 McFarland) of overnight culture of each isolate harvested at the stationary growth phase was made and used for the MIC determinations. Briefly, 100 µl of antibiotic solution was added to 100 µl of broth culture in the 96 – well microtitre plate and incubated overnight at 37°C. The microtitre plates were then observed for the presence or absence of visible growth in the wells (i.e., turbidity of the broth supernatant). Microtitre plates with bacterial species showing visible growth after overnight antibiotic challenge were selected and the residual biofilms determined using the conventional MTP biofilm assay outlined in section 2.4.1. and the absorbance read at 570 nm ( $OD_{570}$ ) using the microtitre plate reader. A positive control was performed for each assay by adding 100 µl 1X PBS to the bacterial broth instead of antibiotic solution. Before reading the absorbance of stained residual biofilms, the microtitre plate reader was blanked using a 33% (v/v) acetic acid. The extent of biofilm inhibition by an antibiotic (also known as percentage (%) inhibition or antibiotic efficacy) was estimated by comparing the  $OD_{570}$  of the residual biofilm of a bacterial species challenged with an antibiotic to that of the same bacterial species without antibiotic challenge (positive control). The formulae for calculating antibiotic efficacy and residual biofilm have been given in section 2.10. Percentage residual biofilms of bacterial species with  $OD_{570} \leq 0.05$  were considered as complete inhibition as  $OD_{570}$  of  $\leq 0.05$  after antibiotic challenge showed no growth on agar after 24 hours of incubation.

#### **2.4.2.2 Minimum bactericidal concentration (MBC) determination**

The MBC is defined as the lowest concentration of antibiotic that can prevent the growth of bacteria after subculture onto media free from antibiotics (Andrews, 2001). The MBCs for all MIC assay plates with no visible growths in the microtitre plates were diluted into  $10^0$ ,  $10^{-1}$ , and  $10^{-2}$ , subcultured on LB agar and incubated overnight at 37°C. The plates were observed for growth after overnight incubation. LB agar plates that showed 0.1% growth (i.e., 99.9% reduction in growth) after antibiotic challenge in comparison with LB agar plates without antibiotic challenge were considered as the MBC.

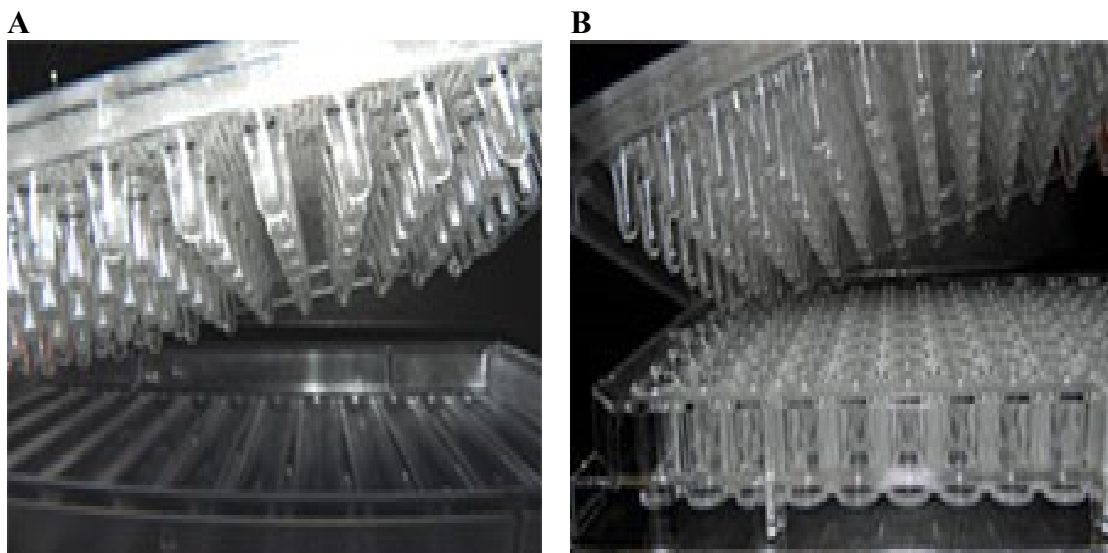


### 2.4.2.3 Minimum biofilm eradication concentration (MBEC) determination

The minimum concentration of antibiotic that can successfully eradicate biofilm formed after 18–24 hours was also determined. The procedure for the determination of MBEC was similar to the one described for MIC determination except that in MBEC determination, antibiotics were added after overnight incubation. The plates were then observed for the presence or absence of biofilm growth by comparing their measured OD<sub>570</sub> with a positive control (bacteria without antibiotic) as previously outlined. Residual biofilm biomass estimation was determined using 0.1% CV as described earlier.

### 2.4.3 The MBEC™ assay

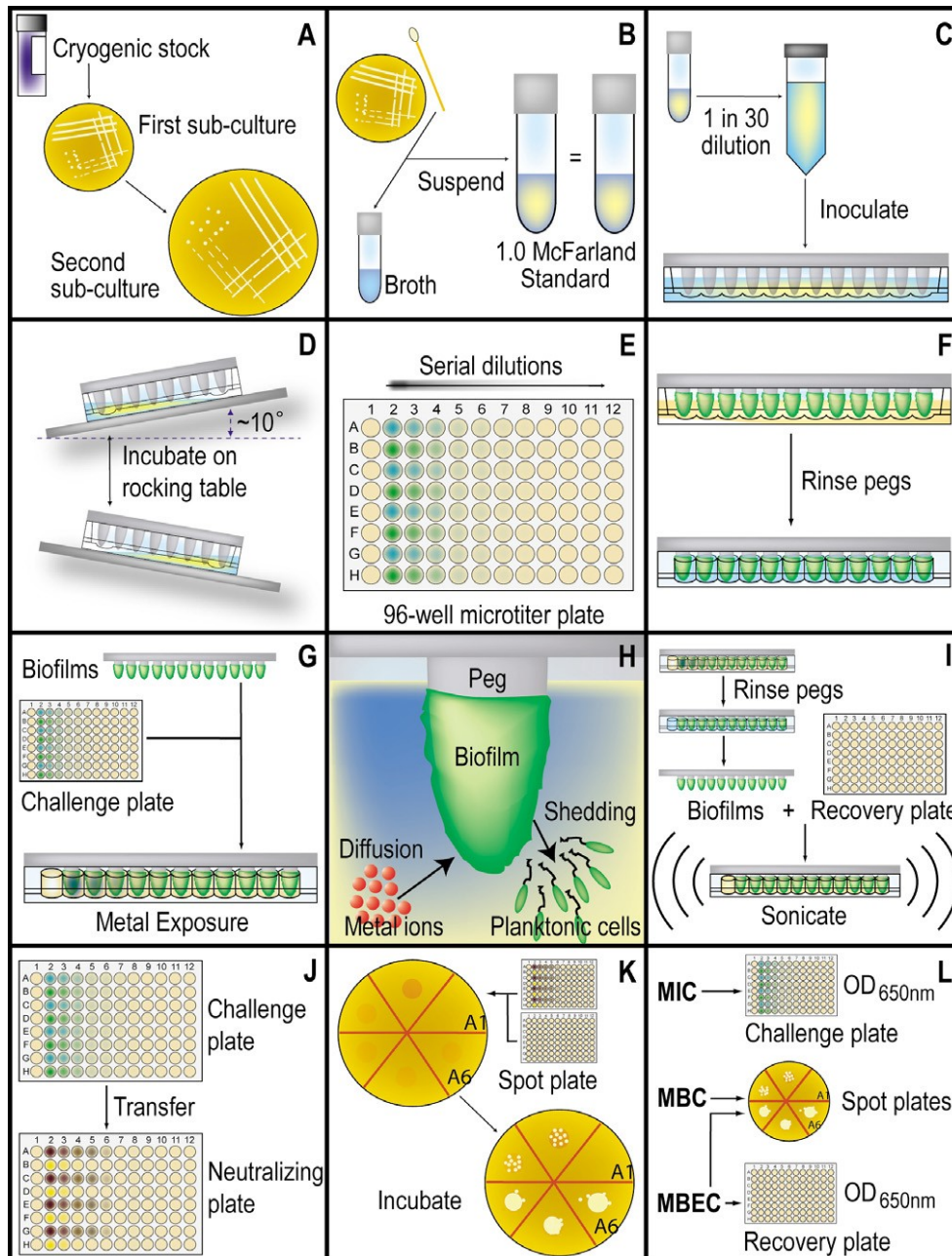
The MBEC™ (Minimum Biofilm Eradication Concentration) assay is a high-throughput assay used to study the effects of antimicrobial agents on microbial biofilms. The MBEC™ assay device also referred to as the MBEC™ Biofilm Inoculator consists of a plastic lid with 96 pegs and a corresponding base; either as a trough base as shown in Figure 2.2A, for high-throughput (HTP) assay or a 96-well base as shown in Figure 2.2B, for Physiology & Genetics (P&G) assay. The device which was initially called the Calgary Biofilm Device was first developed in 1966, by a group of microbiologists at the University of Calgary, Canada to culture and study multiple equivalent biofilms (Ceri *et al.*, 1999).



**Figure 2.2** MBEC™ biofilm inoculator with (A) with a trough base used the MBEC™ HTP assay; (B) with a 96-well base used for the MBEC™ P&G assay. (Image source: MBEC assay procedural manual, version 1.0).

The MBEC™ device has successfully been used to study different microbial species including *E. coli*, *P. aeruginosa*, *S. aureus*, *Candida species.*, *Burkholderia species*, and *Mycobacterium species* (Ceri *et al.*, 1999; Sepandj *et al.*, 2003; Bardouniotis *et al.*, 2003; Harrison *et al.*, 2006; Tomlin *et al.*, 2005).

In this study, both MBEC™ HTP and P&G assays were used to study the efficacy of antibiotics and antibiofilm agents on *K. pneumoniae* and *P. mirabilis* biofilms. The assays were performed according to the manufacturer's instructions and the methods outlined by Ceri *et al.* (1999) and Harrison *et al.* (2004) with some modifications. To prepare the initial inoculum suspensions, *K. pneumoniae* and *P. mirabilis* stored as 50% glycerol (v/v) stocks in 1.5 mL Eppendorf tubes at  $-80^{\circ}\text{C}$  were thawed, plated out on LB agar and incubated aerobically at  $35\text{--}37^{\circ}\text{C}$  overnight. A second subculture was prepared from the primary culture by selecting and plating out two to three pure and single colonies from the overnight LB agar plates onto a second plate and aerobically incubated overnight at  $35\text{--}37^{\circ}\text{C}$ . Single colonies from the second subculture were suspended in LB broth to make a suspension which was adjusted to a 1.0 McFarland standard. This was further diluted 20-fold in fresh LB broth to yield a broth suspension containing approximately  $3.0 \times 10^8$  CFU/mL of bacteria. Bacterial cell density of  $3.0 \times 10^8$  CFU/mL was chosen as the clinically significant microbial count in deep and superficial wounds (Bowler, 2003; Bowler *et al.*, 2001). Twenty-two millilitres of this dilution was inoculated in the MBEC™ HTP assay. The MBEC™ P&G assay plates were inoculated with 150  $\mu\text{L}$  broth culture in each of the 96 wells. The rest of the assay was performed per the manufacturer's instructions. Figure 2.3 provides a summary of the MBEC™ assay. The viable cell densities of the initial inocula for *K. pneumoniae* and *P. mirabilis* were confirmed by total viable cell count on agar.



**Figure 2.3** Overview of the MBECTM HTP assay for the determination of antimicrobial efficacy (Adapted from Harrison *et al.*, 2005). Steps A to L illustrate the stepwise process for the MBEC HTP assay from – **A**. culture of bacterial strains; **B**. inoculum preparation; **C** and **D**. biofilm cultivation and incubation; then through **E**. preparation of antimicrobial challenge plate; **F**. rinsing of pegs; to **J** and **K**. neutralisation and recovery of bacterial strains; and **L**. MIC, MBC and MBEC determinations. In this study, the values for MBC, MIC, and MBEC were determined following absorbance reading at OD<sub>650</sub>. Details of the protocol can be found in Appendix B2.

### 2.4.3.1 Biofilm Growth Check

As part of the MBEC™ assay biofilm growth check (BGC) was performed to estimate the appropriate biofilm growth after overnight cultivation and before antimicrobial challenge. After rinsing the MBEC™ inoculator pegs in normal (sterile) saline, 3 pegs (F12, G12 and H12 in Figure 2.2) were carefully broken off from the lid using sterile pliers, placed in NR media and sonicated on high. The disrupted biofilm after sonication was serially diluted and spot-plated on LB agar for viable cell counting. BGC was expressed as CFU/mL.

### 2.4.3.2 Antimicrobial challenge assay

After the inoculation and overnight incubation of the MBEC™ assay plates (as outlined in section 2.4.3 and Figure 2.3 steps A, B, C and D), the MBEC™ assay plates were subsequently challenged with 2 antibiotics; ceftazidime (CAZ) and levofloxacin (LEV) to determine their MIC, MBC and MBEC. CAZ and LEV were prepared as two – fold dilutions from 5120 µg/mL working solutions. Other antimicrobials such as polymyxin B (PMB) and polymyxin B nonapeptide (PMBN) mentioned in section 2.2.2.3, both at working concentration of 500 µg/mL, were also assayed to determine their MIC and MBC against *K. pneumoniae* and *P. mirabilis*. In separate assays, the combined effects of antibiotics (CAZ and LEV) and antimicrobials (PMB, and PMBN) in inhibiting and eradicating *K. pneumoniae* and *P. mirabilis* biofilms.

Mature biofilms grown on the MBEC™ peg lid were transferred onto a standard flat-bottom 96-well plate (BD Biosciences, UK) containing serially diluted antimicrobials according to the layout in Figure 2.4. In this step, a two-fold serial dilution of each antimicrobial was prepared down the column of each plate using Mueller–Hinton (MH) broth to a final volume of 200 µL. The challenge plates were then incubated at 35 – 37°C for 18 to 24 hours.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	100	100	100	100	100	100	100	100		
B	50	50	50	50	50	50	50	50	50	50		
C	25	25	25	25	25	25	25	25	25	25		SC
D	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5		SC
E	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25		SC
F	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	GC	BGC
G	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	GC	BGC
H	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	GC	BGC

**Figure 2.4** MBEC™ assay antimicrobial challenge plate showing a two-fold serial dilution for a single antimicrobial. The values in the wells represent the percentage (%) concentrations of the antimicrobial diluted from row A1–10 to row H1–10.

### 2.4.3.3 Residual biofilm (Log<sub>10</sub> reduction) estimation

After antimicrobial exposure, the MBEC peg lid was transferred onto a neutraliser–recovery plate containing 200 µL of NR media and let to stand to equilibrate and then sonicated on high for 30 minutes. To determine the amount of biofilm remaining on the peg (Log<sub>10</sub> reduction), neutralised biofilms (in the form of dispersed planktonic cells) in the NR media following sonication were serially diluted (10<sup>0</sup>–10<sup>7</sup>) in 0.9% sterile saline and spot plated on LB agar for *K. pneumoniae* and on MacConkey agar for *P. mirabilis*.

The stepwise formula for calculating the amount of biofilm remaining on pegs after antimicrobial challenge is given below:

Calculate the log density for one peg as follows:  $\text{Log}_{10} (\text{CFU/peg}) = \text{log}_{10} [(X/B) (D)]$

Where: X = mean CFU counted on spot plates, B = volume plated (Ex. 0.02 mL),

D = Dilution

The log density per mm<sup>2</sup> is calculated as follows:

$$\text{Log}_{10} (\text{CFU}/\text{mm}^2) = \text{log}_{10} [(X/B) (V/A) (D) + 1]$$

Where: X = mean CFU counted on spot plates, B = volume plated (Ex. 0.02 mL), V = well volume (0.20 mL), A = peg surface area (46.63 mm<sup>2</sup>), D = Dilution

The Log<sub>10</sub> reduction for each dilution is therefore given as:

$$\text{Log}_{10} \text{ Reduction} = \text{Mean Log}_{10} \text{ Untreated Controls} - \text{Mean Log}_{10} \text{ Treated Pegs}$$

#### **2.4.3.4 MBC determination using MBEC™ HTP assay**

To determine the minimum bactericidal concentration of each antimicrobial used against *K. pneumoniae* and *P. mirabilis* biofilms, 20 µL of broth suspension from each well of the challenge plate was removed and added to 180 µL of fresh LB broth in each well of a new 96-well plate. The plate was covered and incubated under the conditions mentioned earlier. The VersaMax ELISA microplate reader (Molecular Device LLC, USA) was used to determine OD at 650 nm (OD<sub>650</sub>). The MBC value was determined as the lowest antimicrobial concentration that kills 99.9% of the population of dispersed cells shed from the biofilm.

#### **2.4.3.5 MIC determination using MBEC™ HTP assay**

Following biofilm challenge with each antimicrobial, the biofilms on the pegs shed planktonic cells which disperse into the antimicrobial-containing broth suspension during the challenge incubation period. A new non-pegged lid was placed on the 96-well challenge plate base and incubated overnight. Following a suitable period of incubation, the MIC of each antimicrobial was determined by reading the absorbance of the challenge plate at OD<sub>650</sub>. The MIC value was determined as the minimum antimicrobial concentration that inhibits growth of the dispersed cells from the biofilm.

#### 2.4.3.6 MBEC determination using MBEC™ HTP assay

Following the Log<sub>10</sub> reduction step above, the volume of NR media used for the serial dilution is replaced by fresh LB broth. The plate was then covered with a sterile non-pegged lid and incubated overnight at 35–37°C. After the required growth period, the MBEC values were determined for each antimicrobial by reading the absorbance at OD<sub>650</sub>. The MBEC value was determined as the minimum antimicrobial concentration that eradicates the biofilm.

#### 2.4.3.7 Formulae for estimation of biofilm assays

In order to ensure reproducibility and repeatability of the all biofilm assays, coefficient of variation (CV) was calculated for replicate assays (Feiler *et al.*, 2014). Standard deviation, mean values and CV were determined for data generated from all biofilm assays to determine extent of dispersion between resultant OD values (replicates) using the equation;

CV (%) =  $\sigma/\mu \times 100$ ; Where  $\sigma$  is the standard deviation and  $\mu$  is the mean value. CV % was subsequently set at  $\leq 10\%$ .

Percentage efficacy of an antibiotic/antimicrobial (also known as antibiotic activity) and residual biofilm biomass for biofilm inhibition and eradication was determined for all MIC/MBEC replicate assays using the formula;

$$\text{Antibiotic/Antimicrobial efficacy (\%)} = [(A - B) / A] \times 100$$

and,

$$\text{Residual biofilm biomass (\%)} = 100 - \text{Antibiotic/Antimicrobial efficacy (\%)}$$

Where A = absorbance of control (antibiotic free culture), B = absorbance of test sample (bacteria with antibiotic). Percentage inhibition (PI) and percentage reduction (PR) of biofilms by an antibiotic/antimicrobial which can also be defined as the efficacy of that antibiotic/antimicrobial were calculated using the formula;

$$\text{Antibiotic/Antimicrobial efficacy (\%)} = [(A - B) / A] \times 100,$$

or

$$\text{PI/PR} = 100 - \text{Residual biofilm biomass (\%)}$$

#### 2.4.3.8 Biofilm staining and microscopy

*K. pneumoniae* and *P. mirabilis* biofilms were stained and mounted for confocal laser scanning microscopy (CLSM) and epifluorescence microscopy. For CLSM, appropriate pegs (BGC pegs and those challenged with antimicrobials) were broken off from the MBEC™ Biofilm Inoculator by means of sterile pliers. Pegs were placed into empty 96-well plates and stained with LIVE/DEAD® BacLight Bacterial Viability Kit (Molecular Probes, USA) according to manufacturer's instructions. The BacLight kit comprises of a mixture of SYTO® 9 green-fluorescent nucleic acid dye which stains live bacterial cells green and propidium iodide, the red-fluorescent nucleic acid dye which stains dead cells red. In this assay, the standard protocol was modified to stain biofilm pegs. Six microliters of the pre-mixed dyes (SYTO® 9 and propidium iodide) was added to 1 mL of 1X PBS and mixed. The biofilm pegs were placed in the wells of a 96-well plate which were filled with 150 µL of the combined reagent solution. The pegs were stained for 15 – 20 minutes at room temperature in the dark. The pegs were washed with 0.9% sterile saline after staining, transferred into empty wells of a 6-well plate, mounted and examined for biofilm viability using CLSM.

For epifluorescence microscopy 6 µL of a pre-mixed combined reagent solution was added to 1 mL of 1X PBS. Biofilm coverslips placed in the wells of 24-well plates were covered with 250 µL of the combined mixture, covered with aluminium foil and incubated at room temperature for 15 – 20 minutes in the dark. The stained coverslips were washed twice with 1X PBS, mounted with BacLight mounting oil and observed under epifluorescence microscopy.

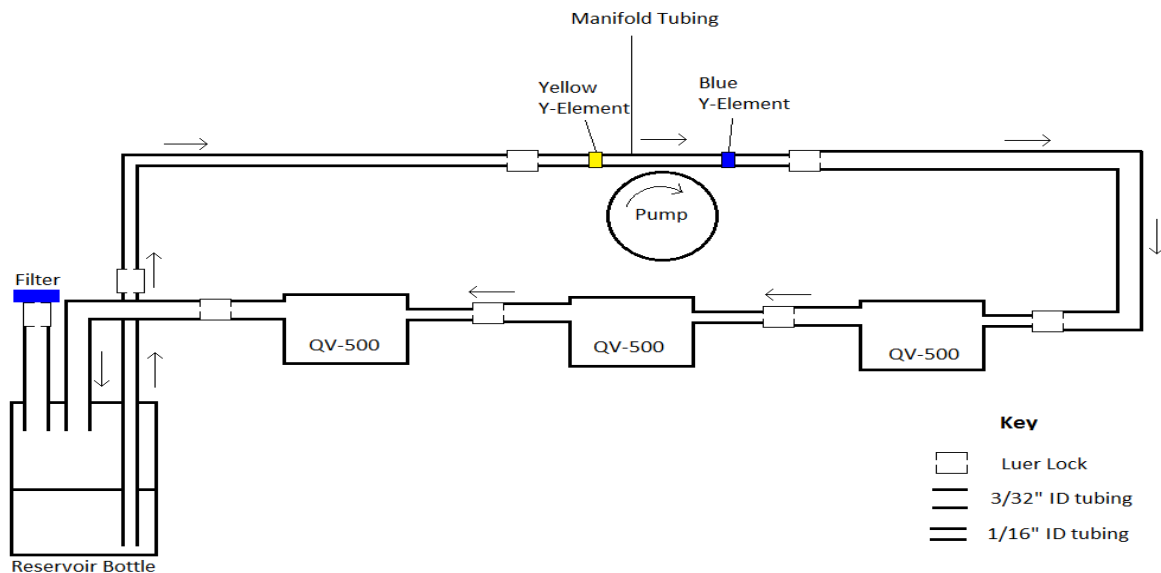
#### 2.4.4 Quasi-Vivo® system

The *Quasi-Vivo*® system (Kirkstall, UK) has been described as a simple, reliable, reproducible and effective cell culture system designed to mimic cellular interactions in living organisms (<http://kirkstall.org/wp-content/uploads/Quasi-Vivo-User-Manual1.pdf>). The *Quasi-Vivo*® system comes in a variety of chambers made to create a continuous flow system to study physiologically relevant biological conditions in *in vitro* cell culture systems. They include the QV500, QV600 and QV900 chamber systems. The system has been tested successfully on a variety of cell lines including human and rat primary hepatocytes, adipocytes, human liver carcinoma (HepG2) human skin and fibroblasts (Pagliari *et al.*,



2014). It has an added advantage of allowing the 3D imaging of cells seeded on glass coverslips using fluorescence microscopy. The *Quasi-Vivo*® system has potential applications in industries such as the pharmaceutical, chemical, cosmetics, research and biotechnology.

It is important to mention that; this is the first time the QV500 chamber has been adapted for growing bacterial biofilms. In this study, the QV500 Chamber system (Figure 2.5) was used to study the effect of exposure of *K. pneumoniae* and *P. mirabilis* biofilms to antimicrobials with time. The assay was set up according to manufacturer’s instructions (User manual for QV500 chamber system, Issue No. 3.0). The QV500 chamber system comprises of a silicone chamber with tubing and connectors, a reservoir bottle, glass coverslips (12 mm diameter), 6 x 22 cm extension tubing with luer connectors. With the exception of extra luer connectors the remaining parts of the QV500 chamber system including the 0.22 µM filter were supplied sterile. The extra luer connectors were sterilised by autoclaving at 121°C for 15 minutes before use.



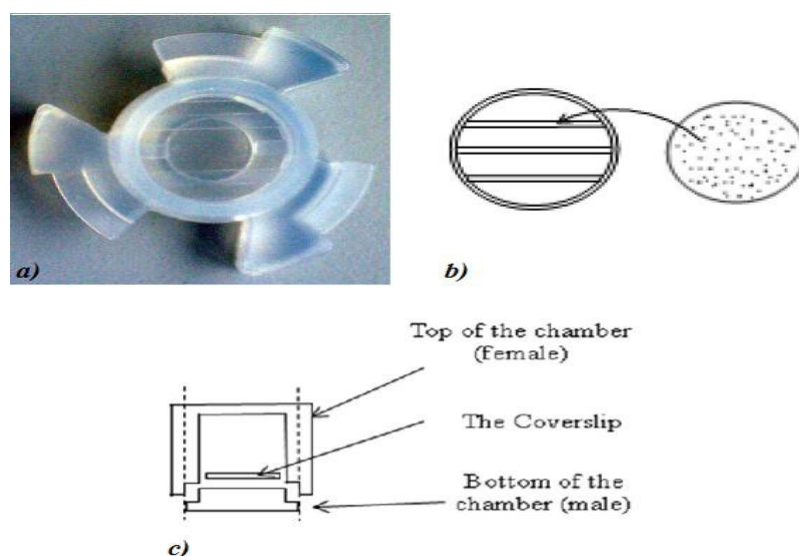
**Figure 2.5** Schematic representation of the complete *Quasi-Vivo*® system set up with three QV500 chambers. (Diagram source: <http://kirkstall.org/wp-content/uploads/Quasi-Vivo-User-Manual1.pdf>).

#### **2.4.4.1 Calibration of the QV500 chamber system**

Before the assay was started, the QV500 system was calibrated according to the method described by the manufacturer. Twenty millilitres (20 mL) of sterile 1X PBS was run through the set up (as illustrated in Figures 2.5 and 2.7) at 4 selected pump speed settings (2, 5, 10 and 15 rpm). After air has been expelled from the system, liquid output from the final chamber was collected over 1 minute. This was repeated 3 times for each speed setting. The flow rate was measured for only the thicker tubing with internal diameter designated as 3/32" ID. This was because it was the collection tubing after the final chamber. The flow rate was calculated as the volume of liquid circulated through the system per minute by the increase in volume of the graduated collection container after 1 minute (Figure B1, Appendix B).

#### **2.4.4.2 Time-dependent biofilm eradication assay**

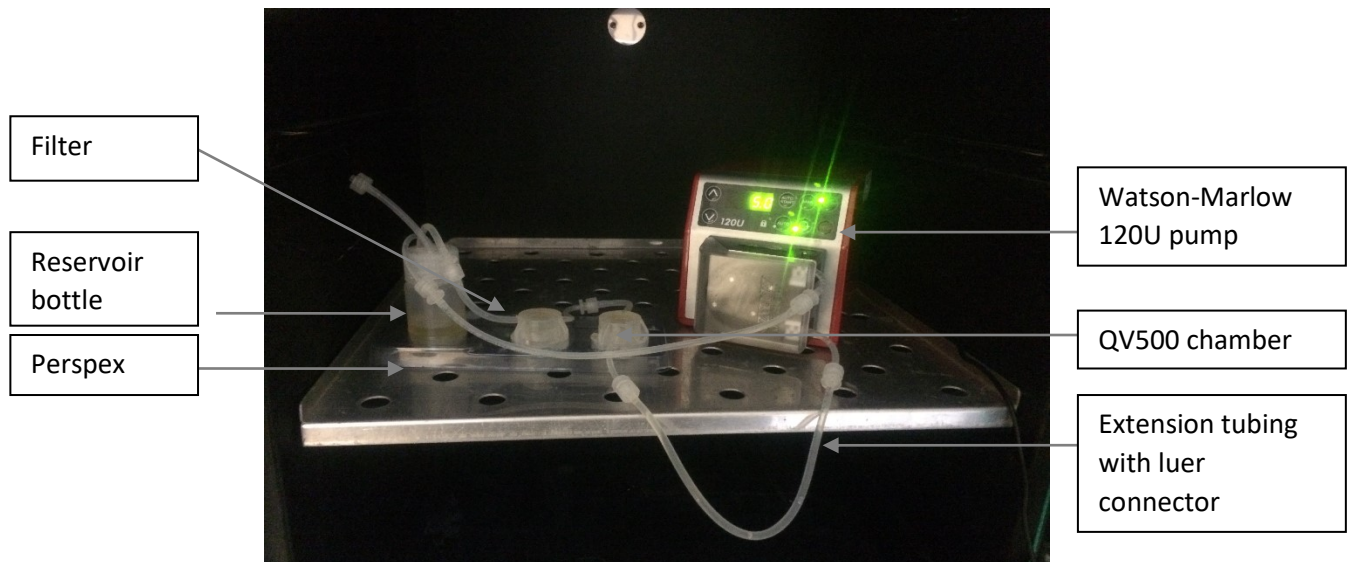
The preparation and cultivation of *K. pneumoniae* and *P. mirabilis* biofilms for the time-dependent assay was similar to that described for cell cultures. Prior to the assay, *K. pneumoniae* and *P. mirabilis* planktonic cells were cultured on the glass coverslips (placed in a receiver vial) to produce biofilms after overnight incubation. The biofilms on the coverslips were aseptically transferred by means of sterile forceps to the bottom of the QV500 chamber (Figures 2.6) containing 1 ml of LB broth. The chambers were connected to the tubings, the reservoir bottle and the pump as illustrated in the set up below in Figure 2.7. The volume of LB broth in the reservoir bottle was 20 mL which could supply each chamber about 4 mL of broth suspension during the run. In separate assays, *K. pneumoniae* and *P. mirabilis* biofilms on coverslips were challenged individually with 20 mL of LB broths containing ceftazidime and levofloxacin at 512 and 5120 µg/mL of respectively. The antibiotic concentrations of 512 and 5120 µg/mL for both ceftazidime and levofloxacin selected and used for the *Quasi-Vivo*® assays were the same concentrations previously used in the biofilm inhibition and eradication assays from sections 2.4.2 to 2.4.3.



**Figure 2.6** QV500 Chamber with (a) visible ridges on the bottom; (b) transfer of the glass coverslip onto the bottom of the chamber and; c) a schematic diagram of the assembled chamber with the coverslip lying on the bottom of the chamber. (Diagram source: User manual for QV500 chamber system, Issue No. 3.0).

The flow rate for the assay was set at 5 rpm (175  $\mu\text{L}/\text{min}$ ) after calibration of the system. This corresponds with the ‘safe range’ (25 – 1000  $\mu\text{L}/\text{min}$ ) established by the manufacturer and as previously stated by Lüdecke *et al.* (2014). The system was placed in an incubator with the peristaltic pump connected and incubated for a period of 24 hours. Samples were collected from the reservoir bottle every hour for the first 5 hours of incubation then at 24 hours. At each sample collection time point, the reservoir bottle was carefully mixed to evenly distribute dispersed bacteria in solution, a one–millilitre (1 mL) sample was aseptically pipetted into a cuvette and the  $\text{OD}_{600}$  measured using a spectrophotometer. To confirm biofilm dispersal, 10  $\mu\text{L}$  of each diluted sample was also spot–plated on LB agar and incubated over overnight. Visible colonies after overnight incubation were counted to determine the CFU/mL for both *K. pneumoniae* and *P. mirabilis* planktonic cells shed from the biofilm on the coverslips. A graph of absorbance ( $\text{OD}_{600}$ ) of samples collected against time was plotted. A control (without antibiotics) for each strain was set up and samples collected at the same time intervals. The assays were repeated at least 3 times and the averages estimated for further analysis.

Residual biofilm on the coverslips after 24 hours of incubation was determined using the LIVE/DEAD® BacLight Bacterial Viability Kit technique with some modifications (section 2.4.3.4).



**Figure 2.7** Set up of the QV500 *Quasi-Vivo*® system connected to a Watson–Marlow 120U/R peristaltic pump.

## 2.5 Isolation of persister cells in Gram–negative DFU isolates

Persister cells are a small population of a biofilm that does not grow nor die in the presence of antibiotics (Keren *et al.*, 2004). They have been suggested to be largely responsible for recurrent infections. Hence they are the other components of a biofilm that would make an otherwise susceptible biofilm resistant to killing by antibiotics even at high concentrations of the antibiotics (Brooun *et al.*, 2000; Lewis, 2005; Spoering and Lewis, 2001).

In this study, the ability of diabetic foot isolates *K. pneumoniae* and *P. mirabilis* to produce persister cells was assessed. Persister cells produced by these strains were then isolated after using 3 methods described by Keren *et al.* (2004) with some modifications. Overnight broth and agar cultures of *K. pneumoniae* and *P. mirabilis* were prepared by diluting thawed cells from –80°C glycerol stocks 1:100 in fresh LB broths and cultured aerobically for up 18–24 hours. Antibiotic (ceftazidime and levofloxacin) concentrations for the challenge assays were selected after MIC determination of each antibiotic in a broth micro–dilution test (British Society for Antibiotic Chemotherapy, 2015; Wiegand *et al.*, 2008). In the broth micro–

dilution assay for MIC determination, two-fold dilutions of ceftazidime and levofloxacin in separate 96-well microtitre plates were prepared and inoculated with *K. pneumoniae* and *P. mirabilis* suspensions corresponding to 0.5 McFarland standard. The plates were incubated overnight at 35 – 37°C under aerobic conditions. MIC was defined as the lowest concentrations of CAZ and LEV that prevented visible growth of *K. pneumoniae* and *P. mirabilis* in the microtitre plates (British Society for Antibiotic Chemotherapy, 2015). MacConkey agar plates were used for *P. mirabilis* CFU confirmation.

### **2.5.1 Time-dependent isolation of persister cells**

Overnight broth cultures of clinical strains were diluted 1:1000 in 20 mL of fresh LB broths in a 125 mL conical flask and cultured by shaking (250 rpm) at 37°C to reach exponential phase. Freshly prepared ceftazidime and levofloxacin were respectively added to 1 mL of each cell in culture tubes to a final concentration of 100 µg/mL and incubated in a shaking (250 rpm) incubator at 37°C for 5 hours. Samples were removed at hourly intervals, serially diluted ( $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$ ) in LB broth and 10 µL spot-plated on LB agar plates to determine their CFUs. Serially diluted antibiotic-free cells were also plated simultaneously for CFU determination as a control.

### **2.5.2 Growth-state dependence assay of persister cells**

A 1:1000 dilution of overnight cultures of *K. pneumoniae* and *P. mirabilis* was made in 20 mL of fresh LB broths and incubated by shaking (250 rpm) at 37°C. At selected time points 1 mL of sample was removed from each culture. Five hundred microlitres was added to 500 µL each of 200 µg/mL ceftazidime and levofloxacin and incubated by shaking (250 rpm) at 37°C for 5 hours. The remaining 500 µL was serially diluted in LB broth and spot-plated on LB agar plates. This was done for 5 different time points and in 3 replicates to rule out false positive results.

### **2.5.3 Persister heritability assay**

In this assay, overnight broth cultures of *K. pneumoniae* and *P. mirabilis* were diluted 1:1000 in 1 mL of fresh LB broth and incubated by shaking (250 rpm) at 37°C for 3 hours. After the incubation period, levofloxacin was added to a final concentration of 100 µg/mL and

incubated at 37°C with shaking (250 rpm) for 3 hours to lyse the cells. Cells remaining after lysis were pelleted by centrifugation at 10,000 x g for 5 minutes at a temperature of 10°C. The pellets were resuspended in 25 mL fresh LB broth and incubated aerobically at 37°C for 18 – 24 hours. Their CFU/mL was determined in order to test for their ability to resist killing by Levofloxacin. The determination of CFU/mL of bacteria has been explained in section 2.3.3. This assay was repeated 4 times on 4 different days and the results compared.

#### **2.5.4 Isolation of persister cells from biofilm**

Antibiotic of choice for this assay was levofloxacin. A two-fold dilution of levofloxacin (5120 µg/mL) was added to 24-hours old *K. pneumoniae* and *P. mirabilis* biofilms in a 96-well conventional microtitre plate. The plates were further incubated at 37°C for 3 hours. The wells were washed with PBS to get rid of planktonic cells in suspension after which the biofilms (with embedded persister cells) were disrupted by rigorous shaking and sonication for 30 minutes. The sonicated-biofilm-forming cells were washed again and centrifuged at 10,000 x g for 5 minutes as described by Keren *et al.* (2004). The pellets were resuspended in fresh PBS, diluted and spot-plated for viability count as previously described in section 2.3.3. Persister cells were recovered from antibiotic agar plates, resuspended in fresh LB broth and inoculated in 96-well plates for crystal violet staining biofilm assay as described above (section 2.4.1). Antibiotic susceptibility testing of 5 antibiotics (ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin) of biofilm-derived *K. pneumoniae* and *P. mirabilis* persister cells was performed and compared to the wild-type using the disc diffusion method described in section 2.3.2.

#### **2.6 Quorum sensing (QS) detection in Gram-negative DFU isolates**

The presence of quorum sensing mechanisms in Gram-negative clinical isolates was tested. A representation of all strains, *K. pneumoniae* and *P. mirabilis* were investigated for their ability to form biofilm using quorum sensing. Three control strains selected for these 3 analyses were *P. aeruginosa* PA01 and *V. harveyi* strains NCIMB 1280 and NCIMB 1872. These control strains belong to the same class of *Gammaproteobacteria* and phylum *Proteobacteria* as the clinical strains under study with similar ancestors and evolutionary relationships (Gao *et al.*, 2009; Williams *et al.*, 2010). The AI-1 – mediated *las/rhl* QS systems in *P. aeruginosa* and three parallel HAI-1, CAI-1 and AI-2 mediated QS circuits in

*V. harveyi* have been well studied (Bassler *et al.*, 1993; Bassler *et al.*, 1994; Domka *et al.*, 2006; Lopez *et al.*, 2010; Patriquin *et al.*, 2008; Xavier and Bassler, 2005b; Wang *et al.*, 2015).

## 2.6.1 Quorum sensing detection by cross-feeding and cross-stimulation assays

### 2.6.1.1 N-acyl-homoserine lactone (AHL) detection using biosensor-reporter system

Diabetic foot isolates were tested for their ability to produce QS molecules using the *lacZ*-fusion reporter gene in an *A. tumefaciens* (NCIMB 14543) broad-spectrum biosensor reporter strain. The production of AHLs by Gram-negative bacteria has previously been reported using different biosensor reporter strains hosts to plasmids with different transcriptional promoter genes fused to the reporter system/gene of interest (Steindler and Venturi, 2007). Table 2.5 provides a summary of some selected reporter systems and the range of AHLs they detect. The biosensors can detect and respond to AHLs ranging from C4-AHL to C16: 1-3-oxo-AHL. AHL detection is normally performed by one of 4 methods; by 'T' – streak analysis on solid media, thin layer chromatography (TLC) analysis on developed (stained) silicone plate, epifluorescence or by quantification (absorbance reading).

**Table 2-5** AHL biosensor reporter system

Host strain	Plasmid sensor	QS system	Reporter system	Reference
<i>A. tumefaciens</i>	pCF218 +pCF372	TraI/R ( <i>A. tumefaciens</i> )	$\beta$ -galactosidase	Zhu <i>et al.</i> (1998)
<i>A. tumefaciens</i>	pMJ258	HyuC/R ( <i>A. tumefaciens</i> )	$\beta$ -galactosidase	Jiwaji (2006)
<i>C. violaceum</i>	CV026	CviI/R ( <i>C. violaceum</i> )	Violacein pigment	McClellan <i>et al.</i> (1997)
<i>E. coli</i>	pSB401	LuxI/R ( <i>V. fischeri</i> )	<i>luxCDABE</i>	Winson <i>et al.</i> (1998a)
<i>E. coli</i>	pSB1075	LasI/R ( <i>P. aeruginosa</i> )	<i>luxCDABE</i>	Winson <i>et al.</i> (1998a)
<i>P. aeruginosa</i>	M71LZ	LasI/R ( <i>P. aeruginosa</i> )	$\beta$ -galactosidase	Dong <i>et al.</i> (2005)
<i>S. meliloti</i>	pJNSinR	SinI/R ( <i>S. meliloti</i> )	$\beta$ -galactosidase	Llamas <i>et al.</i> (2004)
Broad Host	pAS-C8	CepI/R ( <i>B. cepacia</i> )	<i>gfp</i>	Riedel <i>et al.</i> (2001)

### 2.6.1.2 AHL production and detection using the Cross-feeding assay

In this study, a broad host biosensor reporter strain *A. tumefaciens* NCIMB 14543 (also called *A. tumefaciens* RU-AE01 [pMJ258]) with a divergent promoter region separating the 2 reporter genes, *lacZ* and *gus* was used to detect AHL production in diabetic foot clinical isolates. This reporter strain technique which allows the concurrent analyses of promoter sequence-directed transcription and  $\beta$ -galactosidase and  $\beta$ -glucuronidase reporter activities can be used in a wide range of Gram-negative bacteria (Jiwaji, 2006; Jiwaji *et al.*, 2008; Jiwaji and Dorrington, 2009; Matcher *et al.*, 2013). The cross-feeding assay was performed as previously described by Stickler *et al.*, (1998) with few modifications. In the cross-feeding assay the biosensor reporter strain has a *lacZ* gene which is fused together with a transcriptional promoter gene which responds to autoinducer stimulation (Stickler *et al.*, 1998). Quorum sensing molecules produced by a test strain streaked parallel to the biosensor detector strain (in this case, *A. tumefaciens* NCIMB 14543) on an agar plate diffuses through agar and activates the transcription of the *lacZ*-promoter fusion gene. This leads to the production of  $\beta$ -galactosidase enzyme which metabolises the substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) to galactose and an insoluble blue 4-chloro-3-brom-indigo pigment. Thirty-six clinical strains ( $n = 36$ ) were tested for their ability to produce AHLs. They included 22 *P. mirabilis*, 9 *K. pneumoniae*, 1 *K. variicola*, 1 *P. aeruginosa*, 1 *C. koseri*, 1 *E. coli* and 1 *P. stuartii* strains. Prior to the assay, all 36 clinical isolates as well as the *A. tumefaciens* NCIMB 14543 reporter (control) strain were inoculated in LB broths in 96-well microtitre plates and incubated overnight. The wells containing the inoculated *A. tumefaciens* NCIMB 14543 reporter strain were supplemented with 0.05  $\mu$ g/L kanamycin before incubation. This is because the *A. tumefaciens* NCIMB 14543 reporter strain is resistant to kanamycin and hence the addition of 0.05  $\mu$ g/L kanamycin was selective for its growth while preventing the growth of contaminants present in the suspension.

Biofilms produced after overnight incubation were washed once and then resuspended in 100  $\mu$ L of 1X PBS to produce a suspension of biofilm extracts corresponding to 0.5 McFarland standard. X-gal stock solution was also prepared by dissolving 200 mg of X-gal powder (VWR, UK) in 10 mL of dimethyl-sulfoxide (Sigma-Aldrich, UK). The X-gal stock solution was stored at  $-20^{\circ}\text{C}$  in a bottle covered with aluminium foil to protect it against light when not in use. LB agar plates were then covered with 40  $\mu$ L of 20 mg/mL X-gal solution. The



agar plates were left to dry after which the AHL reporter strain and the biofilm extracts of each clinical isolate were streaked across each plate about 1 cm apart and parallel to each other. The plates were incubated at 35–37°C overnight. The plates were observed after 24 hours for evidence of AHL production which was shown by blue colonies for positive results and cream colonies for negative results. *A. tumefaciens* NCIMB 14543 streaked next to itself was used as a negative control. Clinical test strains that stimulated *A. tumefaciens* NCIMB 14543 to produce blue colonies in a positive assay were classified as AHL producers. Clinical test strains that could not stimulate *A. tumefaciens* NCIMB 14543 to produce blue colonies were classified as non-AHL producers.

### **2.6.1.3 Nitrosoguanidine mutagenesis of *V. harveyi* NCIMB 1280**

This assay was performed to determine the ability of diabetic foot isolates to respond to exogenous addition of autoinducer molecules (AI-2) before and after induced mutagenesis. The *V. harveyi* strain NCIMB 1280 has been genetically modified to only respond to AI-2 stimulation through the expression of the *luxICDABEG* operon (confirmed by PCR in section 2.8.5). Prior to the cross-stimulation assay, the NCIMB 1280 strain was subjected to site-directed mutagenesis of the *luxQ* locus using a chemical mutagen called N-methyl-N'-nitro-N-nitrosoguanidine (NTG). NTG can induce GC to AT transitions in the DNA of bacteria and yeast cells (Müller *et al.*, 1978, Siripong *et al.*, 2014) to produce phenotypic variants of the wild-type strains that have either a reduced or increased functionalities. In this study, two *V. harveyi* control strains, NCIMB 1280 luminous strain and NCIMB 1872 aldehyde mutant (i.e., the non-luminous mutant of the wild-type *V. harveyi* strain without the fatty acid reductase complex (*luxCDE*) and could not produce light even after the exogenous addition of long chain aldehyde substrate such as n-decanal) strain were used. Stock solution of 2.5 mg/ml NTG was freshly prepared by dissolving 25 mg of the NTG crystalline powder in 10 mL of 95% ethanol. The NCIMB 1280 strain was mutagenized according to the modified method described by Bassler *et al.* (1994). The 2 strains (mutagenized and wild-type) were cultured in LM broth (Table 2.3) at 30°C overnight to an OD<sub>600</sub> of 1.0. Two representative clinical strains, *K. pneumoniae* and *P. mirabilis* in LB broths were also cultured overnight at 35–37°C in LB broths. The control strains were subcultured in LM broth by adding 2.5 mL of the overnight cultures to 50 mL of fresh LM broth and incubated overnight. Five (5) millilitres of each broth culture was pipetted into 10 separate 15 mL centrifuge tubes and 10

$\mu\text{L}$  of 2.5 mg/mL NTG was added. The resultant cultures were incubated for 10 mins at room temperature, pelleted by centrifugation at  $12,000 \times g$  for 10 mins and then resuspended again in fresh LM broth. The cultures were then incubated at  $30^\circ\text{C}$  overnight with agitation. After overnight incubation, the NCIMB 1280 cells were then screened for mutations by plating out 1:10 dilutions of the broth cultures on LM agar to look for dark and dim mutants in a dark room. Putative dark and dim mutant strains were subsequently designated as *V. harveyi* NCIMB 1280<sub>d</sub>.

#### **2.6.1.4 Cross-stimulation assay**

In this assay both wild-type and putative dim and dark mutants of *V. harveyi* NCIMB 1280 as well as the aldehyde mutant NCIMB 1872 strains were tested for their ability to produce and respond to exogenously added autoinducer (AI-2) molecules using the method described by Bassler *et al.* (1994) with some modifications. The cross-stimulation assay involved the induction of bioluminescence in a *V. harveyi* reporter strain through the production and diffusion of an exogenous AI-2 from a test strain streaked in close proximity to the *V. harveyi* reporter strain (Bassler *et al.*, 1994). A positive test resulted in the observation of luminescence at the edges of the reporter strain close to the AI-2 donor. Expression of luminescence was by exposure of the agar plate to X-ray film or in a dark film development room. Biofilm extracts (as described above) of both control strains (wild-type *V. harveyi* NCIMB 1280, *V. harveyi* NCIMB 1280<sub>d</sub> and aldehyde mutant *V. harveyi* NCIMB 1872 strains) and *K. pneumoniae* and *P. mirabilis* clinical isolates were prepared in sea-water broth and LB broth respectively with final suspensions in 1X PBS adjusted to 0.5 McFarland standard. Control cells were applied onto the centre of sea-water agar (SWA) and L-marine agar plates using sterile cotton swabs to make a 2-cm diameter inoculum. The plates were incubated at  $30^\circ\text{C}$  for 4 hours to dry after which the clinical strains were radially streaked from the edges of the petri dishes to the edges of the control strains in the middle of the agar plates. A second set of LB assay was set up using *A. tumefaciens* 14543 as a negative control and *K. pneumoniae* and *P. mirabilis* as AI-2 donors. In this assay, biofilm extracts were inoculated and streaked on LB agar supplemented with  $0.05 \mu\text{g/mL}$  of kanamycin. The plates were incubated between 8 and 16 hours and then observed for luminescence expression.

## 2.6.2 Bioluminescence assay

The ability of diabetic foot isolates to induce luminescence in *V. harveyi* strain in a co-culture was tested using the bioluminescence assay previously described by Bassler *et al.* (1994). Cell-free culture supernatants were initially prepared from all strains. Both control and clinical strains were grown in AB medium (Table A2, Appendix A) overnight at 30°C in a shaking incubator. Control strains for this assay included *V. harveyi* strains NCIMB 1280, NCIMB 1280<sub>d</sub> and NCIMB 1872. The AB medium used in this assay was the ATCC 2746 autoinducer bioassay (AB) medium. Overnight cultures of the *V. harveyi* strains were diluted 1:5000 into fresh AB medium and incubated again. When the bacteria achieved growth of about 1.0 at OD<sub>600</sub>, they were placed on ice and centrifuged at 5000 x g for 10 minutes. Their supernatants were filtered with a 0.22 µm filters and stored at -20°C. Fresh stocks were prepared every two weeks.

Cell-free culture supernatants from *K. pneumoniae* and *P. mirabilis* overnight cultures were prepared using the method described by Han *et al.* (2010) with some modifications. Ten millilitres (10 mL) of overnight cultures of the clinical strains were centrifuged at 5000 x g for 10 minutes to collect cells and resuspended in 10 mL of fresh AB medium. One-in-one-hundred (1:100) dilutions of broth cultures were prepared by inoculating 500 µL of the resuspended cells into 50 mL of fresh AB medium, which were incubated overnight at 30°C. The growth of the cultures was monitored using a spectrophotometer until it reached 1.0 at OD<sub>600</sub>. The cultures were then centrifuged at 5000 x g for 10 minutes to settle the cells at the bottom of the tubes. The supernatants were then filtered using 0.22 µm filter and the cell-free supernatant stored at -20°C for further analysis.

The bioluminescence plates were set up in 96-well plates at 10% (v/v) by adding 10 µL of cell-free supernatant of clinical strains to 90 µL of *V. harveyi* controls. Positive control wells were made of cell-free supernatants of *Vibrio harveyi* NCIMB 1280 strain and cell-free supernatants of 2 known AI-2 producers; 028b *K. pneumoniae* and 005a *P. mirabilis*. Negative control wells were made up of 90 µL of fresh AB medium and 10 µL cell-free *V. harveyi* NCIMB 1280 or *V. harveyi* NCIMB 1872 (as the reference strains were sensors but not AI-2 producers). Similar to the bioluminescence batch culture mentioned above in this section, a second batch culture assay was set up to determine fold induction of

bioluminescence of *V. harveyi* NCIMB 1280. In this assay, cell-free culture supernatants of 028b *K. pneumoniae* and 005 *P. mirabilis* were used as sources of exogenous AI-2 for light production in *V. harveyi*. To one set of the assay, a commercially prepared AI-1; N-(3-hydroxybutanoyl) homoserine lactone was also added at 1 µg/mL in the presence of AI-2 to determine their combined effect on fold induction of bioluminescence. Luminescence was measured for the first 5 hours and then at 18 and 24 hours using the GloMax<sup>®</sup>-Multi Detection system (Promega, UK). The luminescence plate was incubated inside the luminometre plate reader throughout the reading period. The results of bioluminescence were expressed as relative light units (RLU) and fold induction of bioluminescence was given as the ratio of the relative luminescence (in RLU) of the reporter strain (*V. harveyi* NCIMB 1280) cultured in a conditioned AB medium (in the presence of the donor autoinducer) to the that of *V. harveyi* NCIMB 1280 grown in sterile AB medium.

The ability of exogenous AI-2 donated by 028b *K. pneumoniae* and 005 *P. mirabilis* to stimulate biofilm formation in *V. harveyi* NCIMB 1280 and its mutagenized counterpart NCIMB 1280<sub>d</sub> was also assessed. Biofilms were set up according to the MBEC<sup>™</sup> HTP assay outlined in section 2.4.3 in the presence of exogenous AI-2 from 028b *K. pneumoniae* and 005 *P. mirabilis* cell-free culture supernatants. BGC was determined and presented as CFU/mL.

### 2.6.3 Quorum sensing inhibitors and antibiotic synergy assays

In these assays, the synergy (i.e., when the effects of an antibiotic and an antimicrobial agent in a combination assay were greater than the sum of their individual effects) between quorum sensing inhibitors such as baicalin hydrate (BH), cinnamaldehyde (CIN) and (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (2(5H)-furanone) listed in Table 2.3 and two antibiotics (ceftazidime and levofloxacin) was evaluated in an attempt to develop a strategy for biofilm inhibition and disruption using the high-throughput Physiology and Genetics (P&G) MBEC<sup>™</sup> assay (Ceri *et al.*, 1999; Conlan *et al.*, 2013; Harrison *et al.*, 2004; Harrison *et al.*, 2005). *Klebsiella pneumoniae* and *Proteus mirabilis* biofilms of initial broth suspensions of 10<sup>8</sup> colony forming units (CFU)/mL, cultivated on the pegs of the MBEC<sup>™</sup> device were challenged with 5120 µg/ml of ceftazidime (CAZ) and levofloxacin (LEV) in a double dilution assay in the presence of 1000 µM of CIN. The assays were also performed in

the presence of BH (2500 µg/mL) and 2(5H)–furanone (250 µg/mL). The MICs and MBCs of baicalin hydrate, cinnamaldehyde and 2(5H)–furanone were determined prior to each combination assay. Working solutions of 25 mg/mL, 10000 µM, and 500 µg/ml for BH, CIN and 2(5H)–furanone respectively were diluted in a two–fold dilution for the determination of MIC and MBC. Sub–inhibitory concentrations for BH, CIN and 2(5H)–furanone were then selected for synergy assays with ceftazidime and levofloxacin. The rest of the assay was carried out as outlined from sections 2.4.2 to 2.4.3.3. The presence and extent of synergy between the antimicrobials, quorum sensing inhibitors and the antibiotics (CAZ and LEV) was determined by calculating the fractional inhibitory concentration (FIC) index (Ghosh *et al.*, 2013; Ruden *et al.*, 2009). FIC index was defined as combined antimicrobial effect of agents’ A and B and calculated as follows:

$$\text{FIC index} = \frac{\text{MIC (A in combination with B)}}{\text{MIC (A alone)}} + \frac{\text{MIC (B in combination with A)}}{\text{MIC (B alone)}}$$

Where A is BH, CIN or 2(5H)–furanone and CAZ or LEV

FIC index values were interpreted using the definitions provided by Ruden *et al.* (2009) as guidelines. Synergy was defined as  $\leq 0.5$ ; FIC index  $> 0.5 < 2.0$  was indicative of “additive effect, and FIC index above 2.0 was indicative of antagonistic effect.

At least 3 replicates of each assay were performed and their mean standard deviations used for statistical analysis. OD<sub>650</sub> values computed for mean standard deviations were  $\leq 10\%$  coefficient of variation.

## 2.7 Glycan–lectin interactions among DFU isolates

During biofilm formation, the attachment of bacteria to each other using their cell–surface appendages is an important step in the perpetuation of the biofilm and subsequent reinfection. This is because it provides structural integrity for the biofilm embedded in the EPS (Lopez *et al.*, 2010). Participating bacteria in a developing biofilm use their cell–surface carbohydrate–binding proteins (lectins) to specifically bind to corresponding proteoglycans, glycolipids or glycoproteins of other members of the biofilm consortium. This cell–cell or cell–matrix attachment can provide a useful diagnostic tool for the identification of bacterial species in biofilms using a panel of lectin that specifically bind to receptor carbohydrate monomers or

oligosaccharides. This attachment has also enabled the characterisation of the different carbohydrates and proteins involved in biofilm development. Panel of plant lectins (Table 2.6) have been employed to target and identify some bacterial cell–surface carbohydrates (Afrough *et al.*, 2007; Munoz *et al.*, 1999; Munoz *et al.*, 2003; Slifkin and Doyle, 1990).

**Table 2–6** Panel of commonly used biotinylated plant lectins. Sources and glycan specificity adapted from (Afrough *et al.*, 2007; Lis and Sharon, 1998).

Lectins	Source	Sugar Specificity
<i>Peanut Agglutinin</i> (PNA)	Peanuts	Galactose
<i>Jacalin</i> (JAC)	Jackfruit seed	Galactose/GalNAc <sup>a</sup>
<i>Erythrina cristagalli</i> (ECA)	<i>Erythrina cristagalli</i> seeds	Galactose/GlcNAc <sup>b</sup>
<i>Sophora japonica</i> (SJA)	Japanese pagoda seeds	GalNAc/Galactose
<i>Ricinus communis Agglutinin I</i> (RCA–I)	Castor bean	GalNAc/Galactose
<i>Griffonia simplicifolia Lectin I</i> (GSL–I)	Griffonia seeds	GalNAc/Galactose
<i>Soybean Agglutinin</i> (SBA)	Soybean	GalNAc
<i>Dolichos biflorus</i> (DBA)	Horse gram seeds	GalNAc
<i>Vicia villosa Lectin</i> (VVA)	Hairy vetch seed	GalNAc
<i>Griffonia simplicifolia Lectin II</i> (GSL–II)	Grifonia seeds	GlcNAc
<i>Wheat Germ Agglutinin</i> (WGA)	Wheat gem	GlcNAc/NANA <sup>c</sup>
<i>Succinylated Wheat Germ Agglutinin</i> (SWGA)	Wheat gem	GlcNAc
<i>Lycopersicon esculentum</i> (LEA)	Tomato fruit	GlcNAc
<i>Solanum tuberosum</i> (STA)	Tomato fruit	GlcNAc/Sialic acid
<i>Lens culinaris Agglutinin</i> (LCA)	Lentil seeds	Glucose/Mannose
<i>Concanavalin A</i> (Con A)	Jack bean	Glucose/Mannose
<i>Pisum sativum Agglutinin</i> (PSA)	Garden pea	Glucose/Mannose
<i>Phaseolus vulgaris Leucoagglutinin</i> (PHA–L)	Red kidney beans	Complex Sugar
<i>Phaseolus vulgaris Erythroglubulin</i> (PHA–E)	Red kidney beans	Complex Sugar
<i>Datura stramonium</i> (DSA)	Thorn apple	LacNAc <sup>d</sup>
<i>Ulex europaeus Agglutinin I</i> (UEA–I)	Furze seed	Fucose
<i>Sambucus nigra</i> (SN)	Elder bark	α –2,6 sialic acid gal

<sup>a</sup> – GalNAc – N–acetyl D–galactosamine

<sup>b</sup> – GlcNAc – N–acetyl D–glucosamine;

<sup>c</sup> – NANA – N–Acetylneuraminic acid

<sup>d</sup> – LacNAc – N–acetyl lactosamine

### 2.7.1 Glycan–lectin analysis of whole cells using the enzyme–linked lectinsorbent assay (ELLA)

Lipopolysaccharides (LPS) are the main components of the outer membranes of Gram–negative bacteria that are responsible for maintaining their structural integrity. In biofilms, LPS embedded in the EPS increases the overall negative charge of the matrix and contributes to the stability and protection of biofilms. The negatively charged LPS neutralises any positively charged chemical attack on the developing biofilm. Gygi *et al.* (1995) demonstrated by gas–liquid chromatography that capsular LPS are rich in galacturonic acid and galactosamine monomers. This study employed the optimised method developed by Dharod (2010) with some modifications. This method helps to detect glycans immobilised in the EPS of Gram–negative biofilms by the use of lectins that specifically detect and bind to corresponding sugar monomers. Representative clinical strains used in this assay were *K. pneumoniae* and *P. mirabilis*. A panel of 6 lectins (PNA, JAC, PHA–L, SJA, RCA–I and Con A) in a kit purchased from Vector Laboratories (UK) were selected for this assay as they have high specificity for affinity binding to galactose and galacturonic acid containing–carbohydrates.

Biofilm extracts of the control and clinical strains were prepared as previously mentioned. The final suspension in PBS of OD<sub>600</sub> 0.3 – 0.5 was used to inoculate wells of 96–well microtitre plates and incubated overnight at 35–37°C. To prepare the enzyme extracts, overnight broth cultures of all strains in 15 mL centrifuge tubes were pelleted and the supernatant discarded. The pellets were resuspended in 1.5 mL PE–LB and vortexed for some few seconds. The tubes were incubated for 30 minutes at room temperature and centrifuged for 5 minutes at 10,000 rpm. The supernatants were then pipetted into clean 1.5 mL Eppendorf tubes and labelled as enzyme extracts.

Wells of 96–well plates were inoculated with 100 µL of biofilm PBS suspensions of biofilm extracts. Two controls, negative and positive were set up for each strain. For the negative control, 100 µL of PE–LB was used instead. A known *S. aureus* strain which specifically binds to Con A was used as a positive control (Slifkin and Doyle, 1990). The plates were left in the fridge overnight at 4°C. Unbound cells were removed by carefully pipetting them out. The wells were further washed with 200 µL of PBS to remove other contaminants on a

shaker for 10 minutes. The bound cells were then blocked with 200  $\mu$ L of 5% (w/v) albumin and incubated at room temperature for 1 hour. The blocking agent (5% (w/v) albumin) was discarded after which 100  $\mu$ L of the appropriate diluted biotinylated lectins of concentration 0.6  $\mu$ g/mL were added. The plates were then incubated at room temperature and in the dark for 1 hour. The contents were discarded and the plates washed twice with PBS for 5 minutes to remove unbound lectins. A 1:1000 dilution of streptavidin–alkaline phosphatase was made and 100  $\mu$ L added to the wells and incubated at room temperature for an hour. The contents of the plates were discarded, washed twice with PBS and 100  $\mu$ L of 1 mg/mL of paranitrophenol–phosphate (PNP) added. PNP was prepared by diluting 10 mg of the crystalline powder in 10 mL of 100 mM Tris–HCl at pH of 8.0–8.5. The plates were then incubated at room temperature in the dark for an hour. Fifty microlitres of 3 M NaOH was then added to stop the reaction. The absorbance of the reaction was read using a microtiter plate reader at 405 nm.

### **2.7.2 EPS staining of *in vitro* biofilms with Calcofluor white and ethidium bromide**

In this study, epifluorescence microscopy was used to target and visualise the major components of biofilms such as polysaccharides and DNA in the EPS to qualitatively estimate the production of EPS and confirm biofilm formation. Representative strains *K. pneumoniae* and *P. mirabilis* were grown on coverslips in a 24–well plate to produce biofilms as described in section 2.4.4.2. To demonstrate the presence of the EPS in a mature biofilm, the coverslips were stained with ethidium bromide (Sigma, UK) and Calcofluor white (Sigma, UK) using the method described by Davis *et al.* (2007). Ethidium bromide is a nucleic acid stain which inserts itself between double stranded DNA stains bacterial cells red under fluorescent light (Davies *et al.*, 2007). Calcofluor white on the other hand specifically helps in the identification of polysaccharides by staining 1,4–linked polymers (Davies *et al.*, 2007). After overnight incubation, the planktonic cells were discarded and the coverslips with biofilms fixed with 2.5% formalin for 45 minutes. The biofilms were then stained with 0.5 mg/L of ethidium bromide for 15 minutes, rinsed with 1X PBS and then stained with 0.1% Calcofluor white for 15 minutes. The stained biofilms were visualised by a Leica epifluorescence microscope (Leica Microsystems, UK) with an attached image viewer. The



EPS was stained blue by Calcofluor white and the nucleic acids were stained red by ethidium bromide.

### **2.7.3 EPS staining of *in vitro* biofilms with Congo red and Ziehl carbol–fuchsin**

A second EPS staining method described by Serralta *et al.* (2001) was used to stain polysaccharides in the EPS. In this assay, the quaternary salt, cetylpyridinium chloride first precipitates the polysaccharides in the EPS which is then stained orange/pink by Congo red. The second stain, Ziehl carbol–fuchsin then stains the bacterial cells purple/red. *K pneumoniae* and *P. mirabilis* biofilms were grown on coverslips in a 24–well plate as previously described. After discarding the planktonic cells from the overnight cultures, the developed biofilms on the coverslips were first covered with 10 mM cetylpyridinium chloride solution and air–dried for 20 minutes. The coverslips were further fixed by gentle heating by passing them over a flame of a Bunsen burner and allowed to cool down. Coverslips with the fixed biofilms were then stained with a 2:1 mixture of Congo red solution and 10% (v/v) Tween 80 for 15 minutes, rinsed with distilled water and then stained with 10% (v/v) Ziehl carbol–fuchsin for 6 minutes. Coverslips were rinsed with distilled water, dried at 37°C and then visualised with the Leica epifluorescence microscope. The polysaccharides in the EPS stained orange with Congo red and the bacterial cells stained purple by Ziehl carbol–fuchsin.

## **2.8 Molecular biology and *in silico* studies of DFU isolates**

### **2.8.1 DNA extraction**

DNA of all 50 clinical isolates was extracted using a method described by Millar *et al.* (2000). In this method, 0.5 mL of overnight broth culture was pipetted into 1.5 mL Eppendorf tubes and centrifuged at 13,000 X g for 5 minutes. The pellet was resuspended in TE buffer and incubated at 95–100°C in a heat block for 15 minutes. The suspension was then centrifuged at 13,000 X g for 15 minutes to pellet the thermally denatured bacteria and the resultant supernatant containing the extracted DNA transferred into a sterile 1.5 mL Eppendorf tube and stored at –20°C for future use.

## 2.8.2 Primer design, PCR and agarose gel electrophoresis

All primers used in this study were designed using Primer3 software (Koressaar and Remm, 2007; Untergrasser *et al.*, 2012). All primers used in this study were synthesised by Eurofins MWG Operon, Germany. Unless otherwise stated, all PCR amplifications were performed with the Peltier thermal cycler, DNA Engine®, BIO–RAD using the *Taq* PCR Master Mix kit (Qiagen, Crawley, UK).

All PCR amplified products were resolved on 1% (w/v) agarose gel in 1X TAE buffer. Agarose gels were prepared from agarose crystalline powder purchased from Fisher Scientific Ltd (Loughborough, UK). PCR amplicons resolved on agarose gels were stained with ethidium bromide and subsequently visualised under ultra violet (UV) light using a transilluminator.

## 2.8.3 PCR/gel purification and DNA sequencing

All PCR amplicons that showed single clear bands upon agarose gel electrophoresis were purified following PCR amplification. Gel extraction/clean–up was performed when more than one band was present following agarose gel electrophoresis. All PCR products were purified using QIAquick PCR purification or Gel Extraction kits (Qiagen, Crawley, UK) following the manufacturer’s instructions. In this assay, the initial buffer, PB or QC (solubilising and binding buffer) was added to the PCR amplicons to provide the optimal salt concentration and low pH and enhance the negatively charged DNA to adsorb to the positively charged silica–gel membrane in the QIAquick spin column. Contaminants in solution were then eluted by high speed centrifugation (13,000 rpm) through the column. Buffer PE was then used to thoroughly wash DNA–bound membrane to remove remaining contaminants through centrifugation. The final step of this assay was the elution of DNA using a low concentration Tris buffer (EB). In order to obtain maximum DNA recovery, 40  $\mu$ L of buffer EB was added to the centre of the membrane and the column centrifuged after 1 minute of incubation.

Purified DNA samples were then sequenced by Source Bioscience Ltd (Cambridge, UK) and by GATC Biotech (Germany). DNA sequencing by GATC Biotech (Germany) was performed by the LIGHTRUN™ sequencing technique. In this method, 5  $\mu$ L of purified

DNA and 5 µL appropriate primer of concentration 5µM were premixed before being sent for sequencing. DNA sequences were analysed using bioinformatics tools like BLASTS and MUSCLE to determine their similarities with speciated strains in the databases.

#### 2.8.4 16S rRNA PCR

16S rRNA PCR was performed to confirm the identification of all reference strains. Clinical strains initially identified using the API RapiD 20E were also confirmed by 16S rRNA PCR and DNA sequencing. Three sets of primers (Table 2.7) targeting regions including the V3 and V6 hypervariable regions of the 16S rRNA gene were used for PCR amplification. These primers previously described by Chakravortya *et al.* (2007) are very specific for the conserved 16S rRNA gene sequences in bacteria for identification purposes.

**Table 2–7** Primers and reaction programme for 16S rRNA PCR

Primer name	Sequence	Programme
V3F	5'–CCA g <sup>l</sup> AC TCC TAC GGG AGG CAG – 3'	Initial denaturation – 94°C for 5 minutes, followed by 35 cycles each of 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute then finished with 10 minutes' elongation at 72°C (Chakravortya <i>et al.</i> , 2007)
V3R	5'–CGT ATT ACC GCG GCT GCTG– 3'	
V6F	5' –TCG AtG CAA CGC GAA GAA– 3'	
V6R	5'–ACA TtT CAC aAC ACG AGC TGA CGA– 3'	
339F	5'– ACT CCT ACG GGA GGC AGCAGT–3'	
907R	5 –CCG TCA ATT CMT TTG AGT–3'	

<sup>l</sup> – lowercase base pairs (g, t, and t and a) in primer sequences V3F, V6F and V6R respectively indicating masked effects which did not affect the performance of the primers.

#### 2.8.5 Genotypic characterisation of persister cells and 'wild-type' strains

Attempt was made to determine whether persister cells were genotypic variants of the 'wild-type' (hereafter referred to as controls) strains. PCR was performed by amplifying the gyrase B (*gyrB*) genes of both persister cells and control cells of *K. pneumoniae* and *P. mirabilis*. Persister cells used for *gyrB* PCR amplification were isolated from biofilms in section 2.5.4.

Primers (Table 2.8) were designed to target the *gyrB* genes of both *K. pneumoniae* and *P. mirabilis*.

**Table 2–8** Primers for *gyrB* PCR amplification

Primer name	Sequence	Programme
<i>PGyrB</i> –F	5'–CAG TGA ACA TGC CCC TGC TA–3'	Initial denaturation at 95°C for 5.00 minutes, followed by 30 cycles each of 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 1 minute, then finished with 10 minutes elongation at 72°C.
<i>PGyrB</i> –R	5'–TCA CCA AGC CAC TCA TCC AC–3'	
<i>KGyrB</i> –F	5'–GAG GTG AAA TCA GCG GTG GA–3'	
<i>KGyrB</i> –R	5'–AAG GTC AAC AGC AGG GTA CG–3'	

### 2.8.6 *In silico* studies of quorum sensing activities in DFU isolates

Bioinformatics tools were selected to study the genomes, pathways and other precursors that are responsible for the biosynthesis of quorum sensing molecules and their expressions in diabetic foot isolates. The *in silico* studies were conducted along with molecular biology techniques such as PCR amplification and DNA sequencing to study the expression of quorum sensing genes and the biosynthetic pathways involved in the production of quorum sensing molecules in *K. pneumoniae* and *P. mirabilis*. QS gene expression and QS molecule biosynthesis in *K. pneumoniae* and *P. mirabilis* were compared with previously studied QS systems in *E. coli*, *Salmonella enterica* serovar *typhimurium* and *V. harveyi* to determine similarities and differences between them (Schauder *et al.*, 2001; Winzer *et al.*, 2002; Xavier and Bassler, 2005b). Bioinformatics tools used included the BioCyc database collection (<http://biocyc.org>), National Center for Biotechnology Information (NCBI) databases ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), Universal Protein (UniProt) Resource ([www.uniprot.org](http://www.uniprot.org)). These databases are highly credible sources for accessing high-quality and fully annotated genes, protein sequences and their biosynthetic pathways. The databases also provide tools and other information for studying the functions of these genes, proteins and pathways.

BioCyc database collection is made of a family of databases of 5711 pathways/genome databases (PGDBs) and divided into 3 tiers based on their manual curation. For the purpose of this study, MetaCyc database, which has tools for the illustration of metabolic pathways, chromosomal location of pathway genes, regulation of pathway genes and enzymes from over 2063 organisms (Caspi *et al.*, 2014) was used to study *K. pneumoniae* and *P. mirabilis* QS genes and quorum sensing molecules biosynthesis. This database uses tools that convert text to tables and diagrams for the comprehensive analysis of pathways/genomes data.

NCBI is a division of the United States National Library of Medicine–National Institutes of Health that houses major collections of biomedical databases. It provides extensive information on genomic and proteomic data on several thousands of organisms relevant to biomedicine and biotechnology. NCBI uses bioinformatics tools to access literature and data to analyse structure and function of biologically relevant molecules (<http://www.ncbi.nlm.nih.gov/home/about/mission.shtml>). NCBI Nucleotide, GenBank DNA sequence and BLAST and PubMed databases and other resources were used for the search of QS genes in *K. pneumoniae* and *P. mirabilis* relevant to this study.

UniProt is a comprehensive database that contains high quality and easily accessible literature and data for the analysis of protein sequences and functions. In this study UniProt (Swiss–Prot) was used in searching and confirming annotated protein sequences of QS genes. These protein sequences were further reverse–translated to generate nucleotide sequences that were input into BioCyc and NCBI for further analysis.

Table 2.9 provides a summary of the keywords used in searching for QS and associated genes in the clinical strains identified in the current study.

**Table 2–9** Summary of search for QS and related genes in 3 databases

<b>Search Keywords</b>	<b>Target genes</b>	<b>Database</b>
“Autoinducer–1 in <i>Pseudomonas aeruginosa</i> ”	<i>lasI/rhlR</i>	NCBI, UniProt
“Autoinducer–2 in <i>Vibrio harveyi</i> ”	<i>luxS, luxA, luxLMN</i>	NCBI, UniProt
“Autoinducer–2 in <i>Klebsiella pneumoniae</i> ”	<i>luxS</i>	NCBI, UniProt
“Autoinducer–2 binding–protein in <i>Klebsiella pneumoniae</i> ”	<i>lsrB</i>	NCBI, UniProt
“Autoinducer–2 in <i>Proteus mirabilis</i> ”	<i>luxS</i>	NCBI, UniProt
“Biofilm regulator protein in <i>Proteus mirabilis</i> ”	<i>bssS/bssR</i>	NCBI, UniProt

BioCyc was used to illustrate the biosynthetic pathway responsible for the AI–2 biosynthesis in both test strains. Similar searches were carried out for the control strains. All major enzymes identified in the pathways were selected for PCR amplification.

### **2.8.7 Determination of QS genes by PCR and DNA sequencing**

Primers were designed to target and amplify specific quorum sensing genes in *P. aeruginosa* which are involved in biofilm formation (Table 2.10). They included the autoinducer 1 (AI–1) synthase gene, *lasI*, and the transcriptional regulator gene, *rhlR*, responsible for the expression and regulation of the *las/rhl* QS system in *P. aeruginosa* (Lopez *et al.*, 2010). Primers were also designed for the amplification of QS genes responsible for AI–2 biosynthesis in *K. pneumoniae* and *P. mirabilis* DFU strains as well as in *V. harveyi* NCIMB 1280 and NCIMB 1872 control strains. Other PCR amplifications targeted the biofilm regulatory genes in *P. mirabilis* (*bssS*) and the AI–2 binding–protein (*lsrB* gene) in *K. pneumoniae*.

**Table 2–10** Primers for QS PCR and their respective PCR programmes

Primer name	Sequence	Programme
<i>lasI</i> -F	5'- CAT CTG GGA ACT CAG CCG TT-3'	Initial denaturation at 94°C for 4:50 minutes, followed by 27 cycles each of 94°C for 50 seconds, annealing at 57°C for 30 seconds and elongation at 72°C for 1 minute, then finished with 10 minutes elongation at 72°C (Patriquin <i>et al.</i> , 2008).
<i>lasI</i> -R	5- AGC GTC TGG ATG TCG TTC TG-3'	
<i>rhlR</i> -F	5'- GAA ATG GTG GTC TGG AGC GA-3'	
<i>rhlR</i> -R	5'- TCA CCG TGC TCT CGG AAA TG-3'	
<i>PluxS</i> -F	5'- TTT GCG GGT TTC ATG CGT AA-3'	Initial denaturation at 95°C for 30 seconds, followed by 30 cycles each of 95°C for 30 seconds, annealing at the temperatures* stated below (footnote of table) and elongation at 72°C for 1 minute, then finished with 10 minutes elongation at 72°C.
<i>PluxS</i> -R	5'-TCT TCC ATA GCT GCC TTC CA-3'	
<i>PbssS</i> -F	5'-ACA CAT CCT GTT GTA GGC TGG-3'	
<i>PbssS</i> -R	5'-GCA TTG TCT GGA TGG TGT TCG-3'	
<i>KluxS</i> -F	5'-GTA TCC ACA CCC TGG AGC AC-3'	
<i>KluxS</i> -R	5'-TTC GTC GTT GCT GTT GAT GC-3'	
<i>KlrsB</i> -F	5'-GTG AAA GTC CTG ACC TGG GA-3'	
<i>KlrsB</i> -R	5'-CTT TGG CTT CCT TCA CCC AC-3'	
<i>VluxA</i> -F	5'-TGT TAT GTC GTC GCG GAG-3'	
<i>VluxA</i> -R	5'-ACG GAG GTG ATG TAA GCC AA-3'	
<i>VluxLMN</i> -F	5'-GGC GAC AAG AAA ACA CCA GT-3'	
<i>VluxLMN</i> -R	5'-ATC AAA CAC CAA CAC CGA GC-3'	
<i>VluxS</i> -F	5'-ACC TAC GTT TCA CTG CTC CA-3'	
<i>VluxS</i> -R	5'-TAG TAC GTC TTC CAT CGC GG-3'	

\*Ta (°C) – annealing temperatures for all PCRs (except *lasI* and *rhlR*) in their respective order: 52.3°C, 54.8°C, 56.4°C, 55°C, 55°C, 52.3°C and 55°C.

## 2.9 Effects of wound dressings on biofilms formed by diabetic foot isolates

The aim of this study was to determine the antimicrobial effects of wound dressings in inhibiting biofilm formation or eradicating them. Two sets of assays were performed. First, a selection of wound dressings was tested against the representative strains *K. pneumoniae* and

*P. mirabilis* in their planktonic and biofilm states. The second set of assays evaluated the combined effects of wound dressings and antibiotics on *K. pneumoniae* and *P. mirabilis* biofilms.

### **2.9.1 Inhibition of biofilms by wound dressings (6–well plate assay)**

In this assay, 3 wound dressings with antimicrobial properties namely; Acticoat® (ACT) and Silvercel® (SIL, silver-impregnated), and Medihoney™ Apinate (MDA, honey-impregnated) were tested for their ability to inhibit biofilm formation. According to the manufacturers, the antimicrobial efficacy of silver in ACT and SIL is between 20 – 40 ppm while Medihoney™ is made of 100% Manuka honey. A 4th dressing called Atrauman (ATR) with no antimicrobial properties was used as a control. The assay was performed according to the methods described by Percival *et al.* (2007) and Wright *et al.* (1998). Wound dressings were aseptically cut (in a UV irradiated chamber) into circular shapes (2 cm in diameter). Clinical strains, *K. pneumoniae* and *P. mirabilis*, were inoculated in Mueller–Hinton broth and incubated overnight. Bacterial cell suspension of 10<sup>8</sup> CFU/mL of each strain was prepared and inoculated on each dressing in excess (1 mL) MH broth in a 6–well plate and incubated over 3 time periods; 30 and 60 minutes and 24 hours (Wright *et al.*, (1998). Bacteria were recovered in sodium thioglycolate solution (STS) after their respective incubations times. STS was prepared by adding 0.85% w/v NaCl, 1% v/v Tween 20, and 0.1% w/v sodium thioglycolate to distilled water to make 1 litre solution. The STS bacterial mixture was then vortexed and their OD<sub>600</sub> determined using a spectrophotometer (Jenway, Bibby Scientific Ltd, UK). At least 3 replicates of the assay were performed and the results for OD<sub>600</sub> readings were presented as means (±SEM) at 95% confidence interval. The OD<sub>600</sub> readings were plotted in a graph and percentage inhibition of *K. pneumoniae* and *P. mirabilis* biofilm phenotypes determined at the selected assayed times.

### **2.9.2 Inhibition of biofilm by wound dressings – standard agar method**

This assay was performed to determine the effect of wound dressings on quasi–biofilms using a standard agar technique. Quasi–biofilms are semi-sessile state biofilms that express phenotypes different from clinically relevant biofilm phenotypes and may not express outer membrane proteins when grown on standard nutrient agar (Gilbert *et al.*, 1998). Biofilm extracts of *K. pneumoniae* and *P. mirabilis* were resuspended in 1X PBS to a final



concentration of  $10^8$  CFU/mL. Mueller–Hinton agar (MHA) plates were pre–inoculated with 1 mL of each biofilm extract and incubated at 35–37°C for 30 minutes. Circular shaped dressings which were pre–moistened with sterile PBS were placed in the middle of the plates and incubated overnight at 37°C. After 24 hours, zones of inhibition (ZOIs) developed around each dressing after overnight incubation was measured by means of a pair of callipers or measuring rule. At least 3 replicates of the assay were performed and the results for ZOI measurements were presented as means ( $\pm$ SEM) at 95% confidence interval. A graph of ZOI was plotted to determine the extent of inhibition in the presence of each wound dressing against *K. pneumoniae* and *P. mirabilis* biofilm phenotypes.

### **2.9.3 Combined effects of antimicrobial wound dressings and antibiotics on biofilms**

In this assay, the combined effects of wound dressings (Acticoat<sup>®</sup>, Silvercel<sup>®</sup> and Medihoney<sup>™</sup> Apinate) and antibiotics (ceftazidime and levofloxacin) in the inhibition of *K. pneumoniae* and *P. mirabilis* biofilms were evaluated using the standard agar method. In addition to MHA plates, Kolliphor<sup>®</sup> P 407 (a type of poloxamer hydrogel) gels were used to grow biofilms in the presence of wound dressings and antibiotics. Poloxamer hydrogels and its derivatives such as Kolliphor<sup>®</sup> P 407 gel (used in this study), are made up of a di–block co–polymer of polyoxyethylene and polyoxypropylene (Wirtanen *et al.*, 1998). In aqueous state, they show thermo–reversible gelation and remain liquid at temperatures below 15°C and become robust gels at temperature above 15°C (Gilbert *et al.*, 1998; Wirtanen *et al.*, 1998). The ability of poloxamer gels to support biofilm growth has been explained in section 6.1. This assay was performed according to the method described by Percival *et al.* (2007) with some modifications.

Stock solutions of ceftazidime (CAZ) and levofloxacin (LEV) were prepared and added to biofilm extracts (previously prepared with final broth suspension of  $10^8$  CFU/mL) of *K. pneumoniae* and *P. mirabilis* to final concentrations of 256, 512, 1024 and 5120  $\mu$ g/mL. One microliter volumes of the bacteria–antibiotic suspensions were pre–inoculated on MHA plates and 30% (w/v) Kolliphor<sup>®</sup> P 407 gel plates and incubated at 37°C for 30 minutes to dry. Wound dressings aseptically cut into circular shapes (2 cm–diameters) were placed on the agar and poloxamer gel plates and incubated at 37°C for 24 hours. ZOIs produced by the

3 antibacterial dressings after 24 hours were measured and compared with a control dressing (Atrauman (ATR); with no antibacterial activity). At least 3 replicates of the assay were performed and the results for ZOI measurements were presented as means ( $\pm$ SEM) at 95% confidence interval. A graph of ZOI was plotted to determine the combined inhibitory effect of each wound dressing/antibiotic pair against *K. pneumoniae* and *P. mirabilis* biofilm phenotypes on

## **2.10 Data analysis**

All assays were repeated up to 3 times and their averages determined where necessary. All data for assays performed in this study were statistically analysed using GraphPad Prism (version 5) to determine *P* values and establish correlation between data sets. All graphs were plotted using GraphPad Prism. For biofilm and persister cell isolation assays, linear regression and two-way ANOVA were mostly performed with test of significance limit set at  $p = 0.0001$  and  $0.05$  at 95% confidence interval.

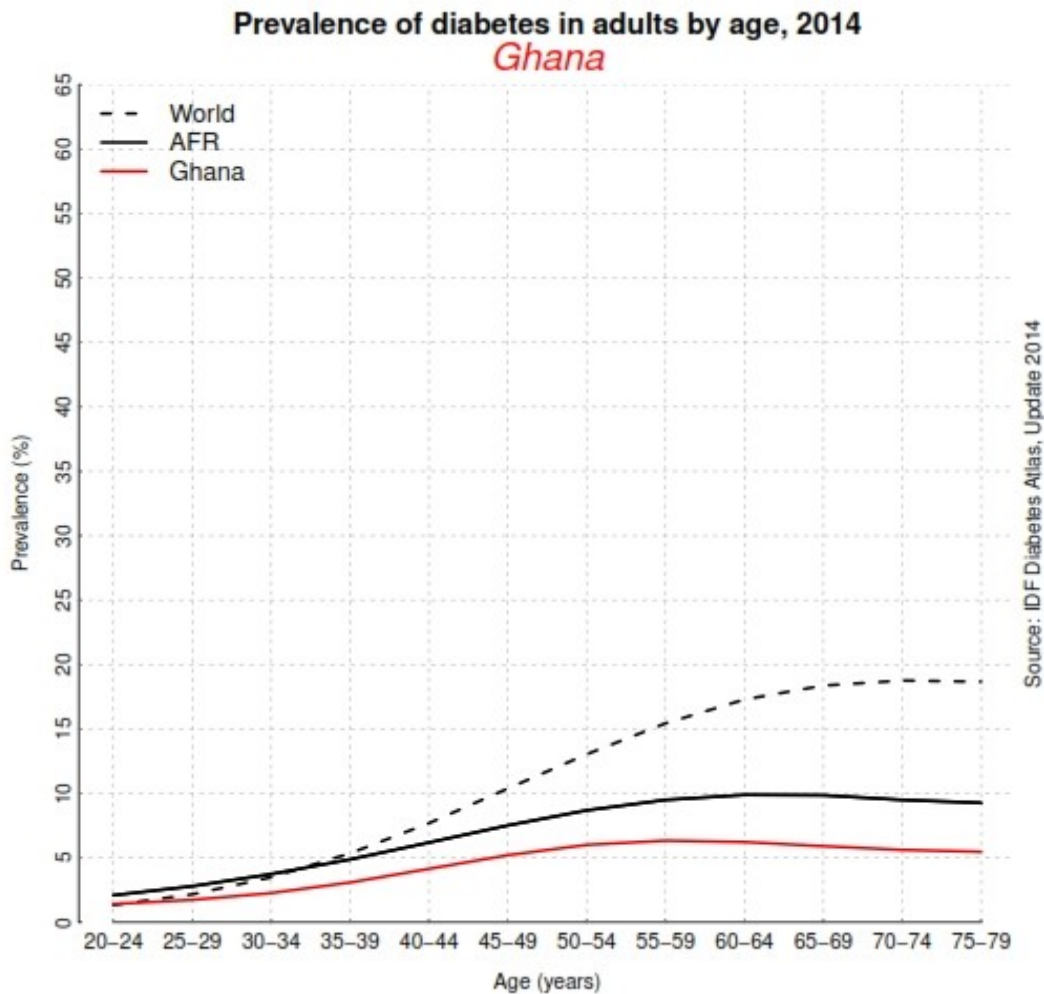
## Chapter 3

# Prevalence studies of diabetes and foot ulcers at the Komfo Anokye Teaching Hospital, Kumasi, Ghana

### 3.1 Introduction

Diabetes mellitus (DM) is one of the major global health concerns with an estimated 415 million people worldwide living with the disease (both types 1 and 2) by the end of 2015 (International Diabetes Federation, 2015). The World Health Organisation (WHO) has predicted an increase in the global trend of the disease with cases estimated to double in the coming years. It has been predicted that about 642 million people would be suffering from DM by the year 2040 (International Diabetes Federation, 2015). The International Diabetes Federation (IDF) has also projected that the number of DM cases in Sub-Saharan Africa is expected to increase from 14.2 million (in 2015) to 34.2 million (in 2040) in line with global predictions (International Diabetes Federation, 2015). However, the major burden of diabetes, about 80% of cases, is borne by DM patients in developing countries (Chen *et al.*, 2012). Also, the incidence/prevalence of the disease among different age groups differ with the number of cases among middle-aged diabetics in developing countries being more than in developed countries (Shaw *et al.*, 2010).

Current data (for the year 2014) on DM in Ghana held by the International Diabetes Federation are based on comparative estimations from data presented by neighbouring countries (Agoudavi *et al.*, 2012; Assah *et al.*, 2011; Ministry of Health Botswana and World Health Organization, 2007; Van Der Sande *et al.*, 1997). These comparative estimations assume that every country and region have the same age profile (Figure 3.1). The comparative and national prevalence estimates were recorded as 3.3 and 3.8% respectively (International Diabetes Federation, 2014). This is because data on DM cases in Ghana have been very scanty since 2002 when the last ‘crude’ prevalence rate (6.3%) was established (Amoah *et al.*, 2002). A recent study by Danquah *et al.*, (2012) characterised type 2 DM among Ghanaians with respect to their socioeconomic status and other complications such as hypertension and albuminuria and suggested hypertension as a complication in most DM cases. It has been estimated that more than two-thirds of people living with diabetes in sub-Saharan Africa are undiagnosed (International Diabetes Federation, 2015). Ghana like other African countries has a higher number of undiagnosed diabetics who are only found at screenings (Evaristo-Neto *et al.*, 2010). The current study presented here is the first to investigate the prevalence of the disease and one of its major complications, diabetic foot ulcer (DFU), in the second largest teaching hospital in Ghana.



**Figure 3.1** Comparison of DM prevalence in Ghana to Africa and rest of world. (Image Source: International Federation of Diabetes (IDF), IDF Diabetes Atlas 6th edition, 2014. Available from: <http://www.idf.org/membership/afr/ghana>. Data used with permission).

The risk of developing foot ulcer increases in people living with advanced diabetes, accompanying complications include peripheral neuropathy, vascular disease, history of ulceration with foot deformities, limited movement of the joint, trauma to the foot and abnormal plantar foot pressure (Boulton, 2004). The development of Charcot foot (also known as neuropathic osteoarthropathy) is a progressive condition accompanied by pathologic fractures with severe destruction to the architecture of the foot and joint dislocation which may result in amputation (Frykberg *et al.*, 2015; Jude and Boulton, 1999). The presentation of DFUs has been well characterised and elaborated in previous studies (Barshes *et al.*, 2013; Boulton, 2015; Gardner *et al.*, 2013; Frykberg *et al.*, 2015; Rathur and Boulton, 2007). The risk of developing DFU among people living with diabetes has been

established as 15–25% (Frykberg *et al.*, 2015). The current global and regional trend of DM suggests an increase in the prevalence of DFU in developing countries. However, the risk of developing a foot ulcer has also been found to be associated with ethnicity (Lavery *et al.*, 1998; Toledano *et al.*, 1993; van Schie *et al.*, 2011). For example some studies carried out in the UK suggest that people of Asian subcontinent origin are less likely to develop foot ulcers and/or undergo amputation than Caucasians (Abbott *et al.*, 2002; Boulton, 2015). With respect to the regional differences in the manifestation of DM and DFU, it is therefore important that prevalence of DM and DFU is established in every country and region in line, hence the Ghanaian study.

It has been estimated that about half of DFU cases are clinically infected at presentation often with complex pathophysiology (Lavery *et al.*, 2006). Asumanu *et al.* (2010) and Lavery *et al.*, (2006) have suggested in separate works that DFU accounts for most diabetes-associated hospital admissions. The definition of the microbiome or the microbial bioburden colonising DFUs is very important in establishing the role of these bacteria in impaired wound healing. Microbial bioburden of chronic wounds is an interplay of 3 factors namely; microbial load, microbial diversity and presence of potential pathogens (Spichler *et al.*, 2015). Traditionally, the first step in estimating the microbial burden of chronic wounds is by culture methods which normally support the growth of non-fastidious bacteria rather than slow-growing ones such as anaerobes (Bowler *et al.*, 2001; Gardner *et al.*, 2013). Some studies have suggested that critical colonisation usually defined as  $>10^5$  colony forming units (CFU) per gram of host tissue is indicative of wound infection that impairs healing (Cutting and White, 2005; Gardner *et al.*, 2009; Richard *et al.*, 2011). However, culture-based assays underestimate the bioburden of DFUs (Bowler *et al.*, 2001; Gardner *et al.*, 2013). It has been established that there is no association between critical colonisation (determined by standard bacterial culture assays), quantitative bacteriology and impaired wound healing and the presence of an infection (Gardner *et al.*, 2013; Spichler *et al.*, 2015). It has rather been suggested that clinical infection is as a result of the presence and interactions of some specific bacteria and their acquisition of virulence factors (Richard *et al.*, 2011).

The use of molecular techniques has revealed that chronic wounds are polymicrobial, with more genetically distinct bacterial population than previously thought to be (Dowd *et al.*, 2008b; Spichler *et al.*, 2015). Genetically distinct bacteria in diabetic foot infections (DFI)

have been found to produce a synergistic community of pathogenic bacteria called a functional equivalent pathogroup (FEP) that maintain the chronicity of wounds (Dowd *et al.* (2008b). The use of molecular biology based methods that incorporate analysis of the 16S rRNA gene has allowed the taxonomic classifications of these pathogroups. The methods include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), PCR, pyrosequencing, and multitarget PCR (Lavigne *et al.*, 2015; Salipante *et al.*, 2013). These methods are superior to culture based assays as they allow the determination of the microbial population and their biodiversity. They also allow the phylogenetic identification of all bacteria present in the microbiome. In one such study, Gardner *et al.* (2013) identified several bacterial species belonging to 13 different phyla including Firmicutes (Gram-positives), Actinobacteria (Gram-positives), Proteobacteria (Gram-negatives), Bacteroidetes (Gram-negative aerobes and anaerobes) and Fusobacteria (Gram-negative anaerobes). However, the bacterial ecology of DFIs has been found to be affected by the interplay of environmental, hygienic and cultural concerns pertaining to a geographical region (Spichler *et al.*, 2015). It is therefore important for healthcare providers to be familiar with the microbial population in their geographical settings with respect to treatment options (Bansal *et al.*, 2008; Spichler *et al.*, 2015).

### 3.2 Aims and Objectives

Diabetes mellitus is a global health issue with significant public health interest. The disease and its associated complications, especially the diabetic foot ulcer, have been well studied. Extensive studies have also been performed to characterise the microbial flora and population in diabetic foot ulcers (Dowd *et al.*, 2008b; Gardner *et al.*, 2013; Lavigne *et al.*, 2015; Salipante *et al.*, 2013; Spichler *et al.*, 2015). However, there is growing need for further studies on DM and DFU among patients in developing countries as they bear the greater burden of the disease in terms of its global distribution. The aim of this chapter is to study the epidemiological pattern of the disease as well as the microbial burden of diabetic foot ulcer among diabetics attending the Diabetes Centre at the Komfo Anokye Teaching Hospital, Kumasi, Ghana. In order to address the epidemiological concerns of DM and DFU in Ghana, the following objectives were considered;

- Determination of the prevalence rates and the demographic distribution of DM and DFU at KATH, Kumasi, Ghana.
- Wound sampling and classification of DFUs among DM patients attending the Diabetes Centre, KATH, Kumasi, Ghana.
- Isolation and identification of the microbiome of DFUs using culture-based and molecular biology techniques such as PCR and DNA sequencing.
- Determination of antibiotic susceptibility patterns of all identified isolates.
- Investigation of the genetic relatedness of clinical isolates using bioinformatics tools.



### 3.3 Wound sampling and classification of diabetic foot ulcers

All participants recruited for this study were sampled once. In all, 356 foot ulcers were sampled among 49166 diabetic patients who attended the Diabetes centre at KATH from January 2011 to December 2014. All samples were characterised (Table 3.1) with respect to the point of collection; whether at the out-patient department (OPD) or in the ward (in-patient). Participants whose samples were collected at the OPD and later admitted into the clinical wards were exempt from subsequent sampling.

**Table 3–1** Number of DFU samples and point of collection

<b>Year</b>	<b>Out-patient cases</b>	<b>In-patient cases</b>	<b>Total</b>
2011	33	21	54
2012	55	32	87
2013	39	74	113
2014	55	47	102
<b>Total</b>	<b>182</b>	<b>174</b>	<b>356</b>

#### 3.3.1 Prevalence of diabetes and foot ulcers at KATH, Kumasi, Ghana

During the period of the current study, a four-year data on the attendance of diabetic patients to the Diabetes centre, KATH was collated with the help of the Statistics Unit, KATH. The corresponding data on diabetic foot ulcers among the diabetic cases were also collated from the Clinical Microbiology Department. The distribution of DM and DFU cases recorded during this period was further categorised in terms of sex to determine the frequency of the cases among male and female subjects (Table 3.2).

The results show that DM was more prevalent in women than in men (72% and 28% respectively). There were at least 42% more women each year who presented with DM than men. Throughout the 4-year period of this study, there were 43% more women who were diagnosed and/or were receiving treatment for DM at the centre than men. With respect to DFU 61% of women were diagnosed compared to 39% of men. With the exception of the year 2014, (when there were 5.8% more women with DFU than men) there were at least 27%

more women with foot ulcers than men from 2011 to 2013. In all, there was 22% more women diagnosed and/or were receiving treatment for foot ulcer than men. It was also observed that the number of DM cases among male subjects decreased steadily over the 4–year period with an average of 2.23% per year and a total of 6.7% at the end of the study period. A similar downward trend was observed among female subjects with an average of 1.74% per year and a total of 5.2% decrease by the end of 2014 (Table 3.1). On the other hand, the number of DFU cases increased steadily over the 4–year period with a total of 21% and 8.8% among male and female subjects respectively. However, the number of DFU cases among female subjects decreased by 14.3% (72 to 54) from 2013 to 2014 (Table 3.2). In all, there was 13.5% increase in the number of DFU cases (both male and female subjects) by the end of the study period.

The difference between the numbers of male and female subjects diagnosed and/or receiving treatment for DM and DFU at KATH was statistically significant ( $p < 0.01$ , at  $\alpha = 0.05$ ) using two–way ANOVA.

**Table 3–2** DM and DFU cases among male and female subjects, KATH from 2011 to 2014

Year	DM Cases			DFU cases		
	Male	Female	Total	Male	Female	Total
2011	4002	9784	13786	19	35	54
2012	3551	9248	12799	31	56	87
2013	3273	8294	11567	41	72	113
2014	3070	7944	11014	48	54	102
<b>Total</b>	13896	35270	<b>49166</b>	139	217	<b>356</b>

According to the 2010 Population and housing Census (Ghana Statistical Services, 2013) the Ashanti region has an estimated population of 4.8 million people representing 19.4% of the total population (24.6 million) of Ghana. Records at the KATH show that an average of 54,000 patients per year attends the hospital. This is about 9000 greater than those in 2006 recorded by the Statistics unit, KATH. From Table 3.3, the average number of DM cases for the four–year period of this study can be calculated as 12292. Therefore, the prevalence rate

of DM among patients who attended KATH from 2011 to 2014 was 22.8%. Adult prevalence rate (excluding the  $\leq 24$  age group) was estimated as 22.1%. Assuming all DM patients who attended the diabetes centre are residents of the Ashanti region, the estimated prevalence rate of DM cases in the region can be calculated as 0.3%. Though this might not be a true reflection of the exact prevalence rate in the region, it is important to mention that, data from KATH covers a wider catchment area of Ghana. The prevalence rate of DFU among the DM cases seen at KATH, Kumasi, Ghana for the four-year period can be estimated as 0.7%.

The number of DM and DFU cases were also considered in terms of the age groups associated with the disease (Tables 3.3 and 3.4). The median age group for both DM and DFU cases was 35–44 years. The mean ages for DM and DFU patients were estimated as 56.3 and 50.3 years respectively. Most of the diabetic patients in Table 3.3 (41.3% of cases) were within the 45–59 age group followed by the  $\geq 60$ s age group (35.6%) and 35–44 years (14.5%). Diabetics belonging to the  $\leq 24$  age group contributed the least number of DM cases (1399) representing 2.8% of cases.

**Table 3–3** Age and sex distribution of DM cases

Year/Sex	Age Groups										Total
	$\leq 24$		25 – 34		35 – 44		45 – 59		$\geq 60$		
	M	F	M	F	M	F	M	F	M	F	
2011	181	285	217	630	575	1423	1619	4065	1410	3381	13786
2012	176	253	254	631	527	1351	1375	3906	1219	3107	12799
2013	129	165	164	462	474	1272	1347	3601	1160	2793	11567
2014	80	129	115	389	379	1108	1148	3228	1348	3090	11014
<b>Total</b>	566	832	750	2112	1955	5154	5489	14800	5137	12371	<b>49166</b>

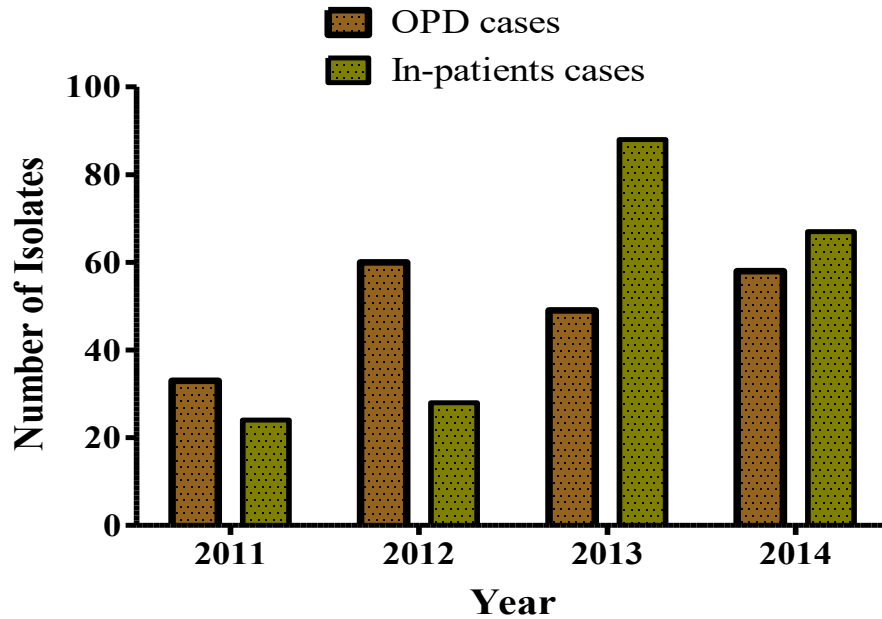
Table 3.4 illustrates that, there were more DFU cases in diabetics of 60 years and above representing 39.9% of cases followed by those between 45–59 years (32.8% of cases). Diabetics likely to have least number of ulcers were those belonging to the  $\leq 24$  age group (2.2% of cases). Interestingly, the number of DFU cases among the  $\geq 60$  male subjects

increased steadily from 7 in 2011 to 9 in 2013. There was however a sharp increase from 9 in 2013 to 24 in 2014. There were however inconsistent trends in the number of DFU cases recorded in the other age groups over the 4-year period of the study.

**Table 3–4** Age and sex distribution of DFU cases

Year/Sex	Age Groups										Total
	≤ 24		25 – 34		35 – 44		45 – 59		≥ 60		
	M	F	M	F	M	F	M	F	M	F	
2011	0	1	2	2	3	4	7	9	7	19	54
2012	0	1	1	2	8	15	14	18	8	20	87
2013	1	4	3	0	3	10	25	27	9	31	113
2014	0	1	6	8	6	16	12	5	24	24	102
<b>Total</b>	1	7	12	12	20	45	58	59	48	94	<b>356</b>

There was no statistically significant difference between the number of in-patient and out-patient cases (Table 3.1) receiving treatment for DM at KATH ( $p = 0.76$ ). However it is clear that the number of OPD to in-patient cases has varied over the last 4 years from a ratio of approximately 2:1 to 1:2 in 2012 and 2013 respectively. In 2011 and 2014, the number of OPD cases was 22.2 and 7.8% (respectively) more than in-patient cases. It is clear that there was no consistent trend in the number cases (OPD and in-patient) sampled each year throughout the 4 year-period of the study. A similar situation was observed when the number of bacterial isolates identified from ODP and in-patient samples was compared (Figure 3.2). There were more isolates (9 and 32) from OPD cases in 2011 and 2012 respectively than from in-patient cases. However, more isolates (39 and 9) were recovered from in-patient cases in 2013 and 2014 respectively than from OPD cases. There was no significant difference ( $p = 0.82$ ) between the number in-patient and OPD cases. Though there were 8 more OPD cases in total than in-patient cases, 7 more bacterial isolates were recovered from in-patient cases than OPD cases.



**Figure 3.2** Number of isolates from OPD and In-patients DFU samples. There was no significant difference between the number of in-patients DFU and OPD cases ( $p = 0.82$ , column factor two-way ANOVA).

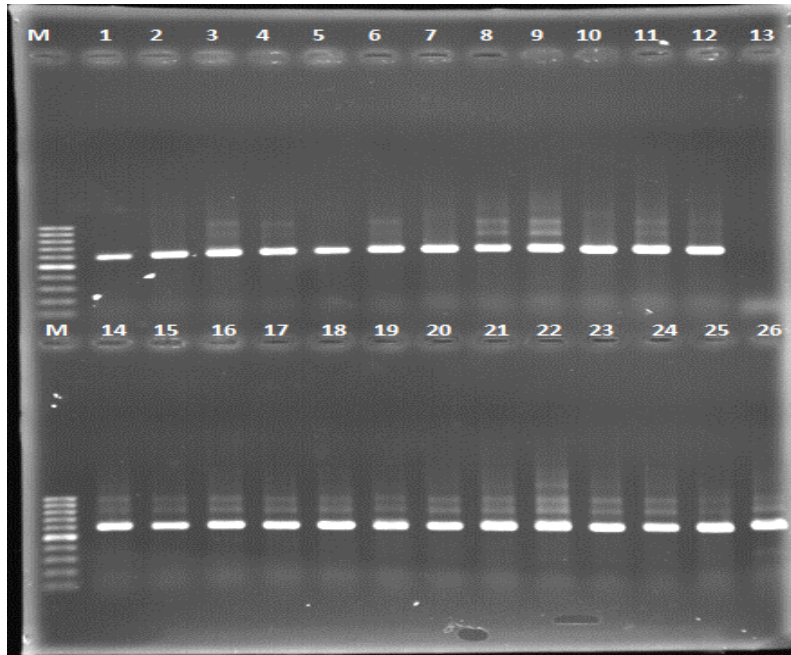
Out of the 356 DFUs sampled throughout the period of the current study, only 38 of them collected between February and April 2013 were further classified according to Wagner’s ulcer classification grade system (Tables 3.5) which is based on the depth of penetration, the presence/absence of gangrene and the degree of tissue necrosis (Wagner, 1987).

**Table 3–5** Distribution of ulcer types and bacterial isolates in 38 DFU

Ulcer grade	Frequency	No. of isolates
Superficial	7	9
Deep	21	29
Abscess osteitis	4	5
Gangrenous foot	5	6
Whole foot	1	1

### 3.4 Identification of clinical isolates and control strains

The presence of bacteria in wound samples was confirmed by microbiological, biochemical and molecular biology techniques as described in section 2.2, chapter 2. Fifty bacterial species (members of the Proteobacteria group) isolated from 38 wound samples collected as part of the current study in Ghana were used as representative strains for further investigations. The speciation of these 50 bacterial strains was confirmed by 16S rRNA PCR and DNA sequencing (Figure 3.3 and Table 3.6). The resultant sequences were aligned with speciated strains in the NCBI databases using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Bacteria identified included *P. mirabilis* (23) representing 46% was the most isolated strain followed by *E. coli* 12 (24%), *Klebsiella* species 6 (12%), *K. pneumoniae* 4 (8%), *P. stuartii* 2 (4%), *P. aeruginosa* 2 (4%) and *C. koseri* 1 (2%). The initial classification of some *Klebsiella* isolates was denoted as *Klebsiella* species as biochemical and 16S rRNA sequencing identified different species. API<sup>®</sup> identification denoted them as *K. oxytoca* as they were the nearest likely identification (based on classification hierarchy). 16S rRNA DNA sequencing identified them as *K. variicola* and *K. pneumoniae* respectively (as highlighted in Table 3.6) after BLAST analysis. Culture-based assays such as the API<sup>®</sup> identification strip tests have been reported to have limitations in the accurate taxonomic identification for *Klebsiella* species (Alves *et al.*, 2006). The API<sup>®</sup> identification system is based on the scoring of a panel of biochemical assays using a numerical system that identifies organisms from among 700 bacterial and fungal species deposited in the API<sup>®</sup>/ID32 databases (<http://www.biomerieux-diagnostics.com/apir-id-strip-range>). The scoring of the API<sup>®</sup> test strip, which is based on positive and negative results of a panel of biochemical tests on the strip, generate a hierarchy of bacterial profiles from “through calculation of identification percentage and typicity index” (<http://www.biomerieux-diagnostics.com/apir-id-strip-range>). One of the limitations of bacterial identification using biochemical assays such as the API<sup>®</sup> system is the fact that several bacteria share similar biochemical profile which lead to taxonomic complexities (Alves *et al.*, 2006; Westbrook *et al.*, 2000). Culture-based methods were able to detect more than one species of bacteria in 63 (17.7% of cases) wound samples. The maximum number of isolates recovered from a single wound sample in this study was 2. The remaining samples produced single species of bacteria each.



**Figure 3.3** PCR amplification of the 16S rRNA V3 – V6 hypervariable regions. Lane M – 100 bp DNA ladder; lanes 1 to 26 with the exception of lane 13 (negative control) represent amplified 586 base pairs partial 16S rRNA sequence of clinical isolates from 017 to 031 in the order shown in Table 3.6.

**Table 3–6** Wagner’s ulcer classification of DFUs and identification of clinical isolates

<b>DFI ID</b>	<b>Description of ulcer</b>	<b>Grade</b>	<b>API® ID</b>	<b>16S rRNA ID</b>
001	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
002	Gangrenous forefoot	4	<i>P. mirabilis</i>	<i>P. mirabilis</i>
003a	Deep	2	<i>C. koseri</i>	<i>C. koseri</i>
003b	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
004	Superficial	1	<i>P. mirabilis</i>	<i>P. mirabilis</i>
005	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
006	Gangrenous forefoot	4	<i>P. mirabilis</i>	<i>P. mirabilis</i>
007	Abscess osteitis	3	<i>P. mirabilis</i>	<i>P. mirabilis</i>
008	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
009	Superficial	1	<i>P. mirabilis</i>	<i>P. mirabilis</i>
010a	Deep	2	<i>E. coli</i>	<i>E. coli</i>
010b	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
011	Superficial	1	<i>P. mirabilis</i>	<i>P. mirabilis</i>
012	Deep	2	<i>E. coli</i>	<i>E. coli</i>
013	Deep	2	<i>E. coli</i>	<i>E. coli</i>
014	Superficial	1	<i>P. mirabilis</i>	<i>P. mirabilis</i>
015	Superficial	1	<i>E. coli</i>	<i>E. coli</i>
016	Superficial	1	<i>P. mirabilis</i>	<i>P. mirabilis</i>
017	Deep	2	<i>E. coli</i>	<i>E. coli</i>
018a	Gangrenous forefoot	4	<i>P. mirabilis</i>	<i>P. mirabilis</i>
018b	Gangrenous forefoot	4	<i>K. oxytoca</i>	<i>K. variicola</i>
019a	Gangrenous forefoot	4	<i>E. coli</i>	<i>E. coli</i>
019b	Gangrenous forefoot	4	<i>P. mirabilis</i>	<i>P. mirabilis</i>
020	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
021a	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
021b	Deep	2	<i>K. oxytoca</i>	<i>K. pneumoniae</i>



**Table 3–6** Wagner’s ulcer classification of DFUs and identification of clinical isolates. Continued.

<b>DFI ID</b>	<b>Description of ulcer</b>	<b>Grade</b>	<b>API® ID</b>	<b>16S rRNA ID</b>
022a	Superficial	1	<i>E. coli</i>	<i>E. coli</i>
022b	Superficial	1	<i>K. oxytoca</i>	<i>K. pneumoniae</i>
023a	Deep	2	<i>K. oxytoca</i>	<i>K. pneumoniae</i>
023b	Deep	2	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>
024	Deep	2	<i>E. coli</i>	<i>E.coli</i>
025a	Deep	2	<i>P. stuartii</i>	<i>P. stuartii</i>
025b	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
026	Deep (toe amputation)	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
027	Deep (foot amputation)	2	<i>K. oxytoca</i>	<i>K. pneumoniae</i>
028a	Abscess osteitis	3	<i>E. coli</i>	<i>E. coli</i>
028b	Abscess osteitis	3	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
029	Abscess osteitis	3	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
030	Abscess osteitis	3	<i>E. coli</i>	<i>E. coli</i>
031	Gangrenous forefoot	4	<i>E. coli</i>	<i>E. coli</i>
032a	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
032b	Deep	2	<i>K. oxytoca</i>	<i>K. pneumoniae</i>
033	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
034a	Deep	2	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
034b	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
035	Deep	2	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
036a	Whole foot (both)	5	<i>E. coli</i>	<i>E. coli</i>
036b	Whole foot (both)	5	<i>P. stuartii</i>	<i>P. stuartii</i>
037	Deep	2	<i>P. mirabilis</i>	<i>P. mirabilis</i>
038	Deep	2	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>

In all 407 bacterial strains (Table 3.7) were isolated from 356 DFU samples from January 2011 to December 2014. Bacteria isolated in Ghana were identified using the API® ID strip range of testing in the Clinical Microbiology department, KATH, Kumasi and the microbiology/molecular biology laboratory, University of Westminster, London. Uncultivable or slow growing bacteria if present in some of the DFU samples collected might have been undetectable by culture-based assay.

**Table 3–7** Distribution of the number of different bacterial species in all 356 DFU samples

<b>Isolate</b>	<b>2011</b>	<b>2012</b>	<b>2013</b>	<b>2014</b>	<b>Total</b>	<b>Freq. (%)</b>
<i>Pseudomonas</i> species	15	32	26	36	109	27
<i>Proteus</i> species.	11	15	40	21	87	21
<i>Escherichia</i> species	9	18	32	26	85	21
<i>Klebsiella</i> species	7	12	21	21	61	15
<i>Staphylococcus</i> species	8	5	10	8	31	8
Others*	7	6	8	13	34	8
<b>Total</b>	<b>57</b>	<b>88</b>	<b>137</b>	<b>125</b>	<b>407</b>	<b>100</b>

\* Others – Other microbes (8.0%) isolated included *Enterobacter* species, *Citrobacter freundii*, *Bacillus* species, *Citrobacter koseri*, *Moraxella catarrhalis*, *Alcaligenes* species, *Bacillus* species, *coliforms*, *yeast cells* and *Providencia stuartii*.

### 3.4.1 Antibiotic susceptibility determination of DFU isolates

The disc diffusion test interpreted using the British Society for Antimicrobial Chemotherapy (BSAC, 2015) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) methods for antimicrobial susceptibility testing indicates that co-trimoxazole (COT) was the least effective antibiotic as 86% (350) isolates were resistant followed by ampicillin/sulbactam 77% (312) and tetracycline 70% (286) respectively (Figure 3.4). Levofloxacin (LEV) was the most effective quinolone as it was effective against 65% (264) of clinical isolates followed by the tazobactam/piperacillin 64% (263) ciprofloxacin 62% (253) respectively. Table 3.8 provides more details on the antibiotic susceptibility testing of all isolates.

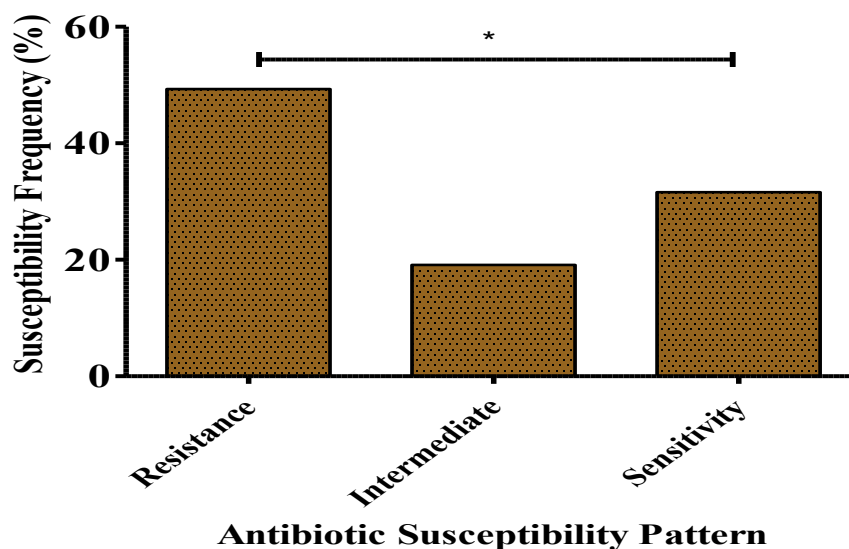
**Table 3–8** Antibiotic susceptibility pattern of all clinical isolates

Antibiotics	Susceptibility patterns of DFU isolates		
	Sensitive	Intermediate	Resistant
Ampicillin/sulbactam, 20 µg <sup>a</sup>	20 (5%)	75 (18%)	312 (77%)
Cotrimoxazole 25 µg	10 (2%)	47 (12%)	350 (86%)
Cefotaxime, 30 µg	60 (15%)	68 (16%)	279 (69%)
Tazobactam/Piperacillin, 100/10 µg	263 (64%)	36 (9%)	108 (27%)
Chloramphenicol, 30 µg	65 (16%)	85 (21%)	257 (63%)
Ciprofloxacin, 5 µg	253 (62%)	33 (8%)	121 (30%)
Tetracycline, 30 µg	23 (6%)	98 (24%)	286 (70%)
Ceftazidime, 30 µg	181 (44%)	89 (22%)	137 (34%)
Ofloxacin, 5 µg	199 (49%)	70 (17%)	138 (34%)
Gentamicin, 10 µg	81 (20%)	139 (34%)	187 (46%)
Amikacin, 30 µg	124 (30%)	162 (40%)	121 (30%)
Levofloxacin, 5 µg	264 (65%)	30 (7%)	113 (28%)

<sup>a</sup> – antibiotic disc concentration.

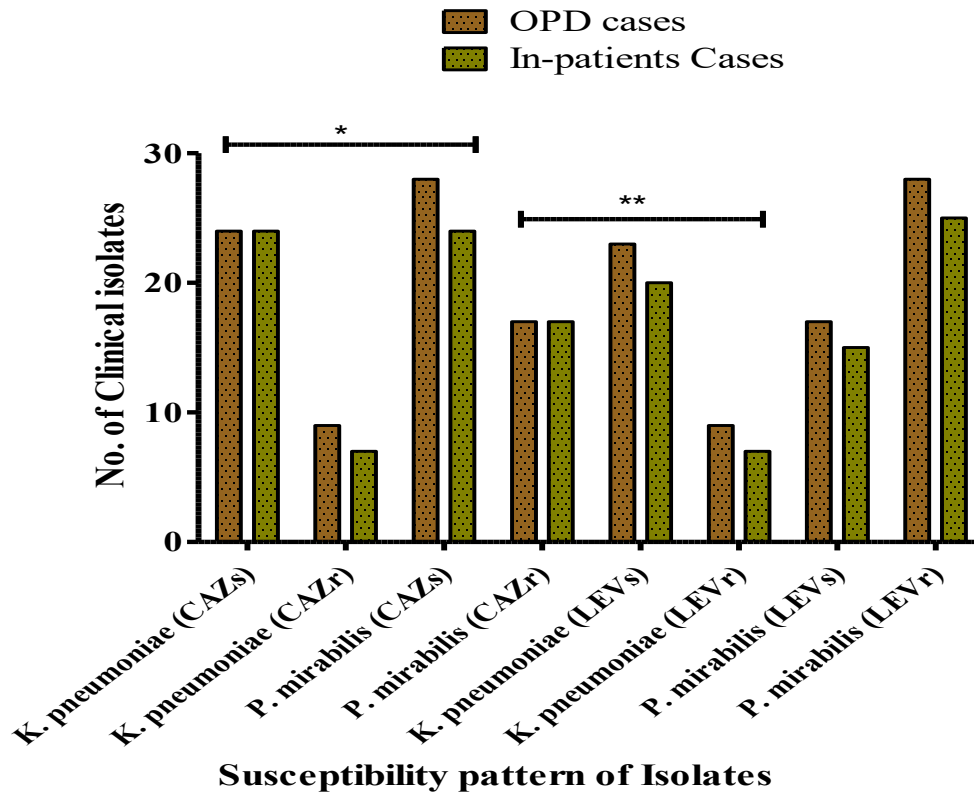
The antibiotic susceptibility pattern in Figure 3.4 shows that there were 17.7% more resistant cases than there were sensitive cases. However, the increase number of resistance cases was not statistically significant ( $p = 0.25$ ) when compared to the number of sensitive cases.

According to British Society for Antimicrobial Chemotherapy, an antibiotic is clinically intermediate when its therapeutic effect is uncertain, can effectively treat an infection by an isolate at body sites where the antibiotic is highly concentrated or can create a buffer zone when administered by preventing small, uncontrolled or other bacterial antibiotic invasive mechanisms from causing major problems (Andrews, 2010; Wootton, 2013).



**Figure 3.4** Antibiotic susceptibility patterns of all DFU isolates. According to Wilcoxon Signed Rank test ( $\alpha = 0.05$ ), the relationship between the antibiotic susceptibility patterns of DFU isolates was statistically insignificant ( $p = 0.25$ ).

Figure 3.5 (below) illustrates the relationship between the susceptibility patterns of two antibiotics against OPD and in-patient strains of *K. pneumoniae* and *P. mirabilis*. In all there were 175 ODP and in-patient *K. pneumoniae* and *P. mirabilis* strains that were sensitive to CAZ and LEV compared to 119 strains that were resistant to CAZ and LEV. There were 7 (5.9%) more OPD strains that were resistant to CAZ and LEV than in-patient strains and the difference was found to be statistically significant ( $p < 0.0001$ ). Similarly, there were 9 (5.1%) more OPD strains of *K. pneumoniae* and *P. mirabilis* that were sensitive to CAZ and LEV than in-patient strains and the difference was found to be statistically significant ( $p < 0.05$ ). From the analysis of the susceptibility patterns of *K. pneumoniae* and *P. mirabilis* test strains to CAZ and LEV, 2 strains namely 028b *K. pneumoniae* and 005 *P. mirabilis* were subsequently selected as representative strains for further investigations in the course of this study. *K. pneumoniae* and *P. mirabilis* were chosen for further studies in order to establish whether their dominance in DFUs samples in the Ghanaian study cohort and relative susceptibility to ceftazidime (an extended beta-lactamase inhibitor) and levofloxacin (a fluoroquinolone with good tissue permeability and DNA topoisomerase and gyrase B inhibitor) make them suitable models to study and understand biofilm formation and quorum sensing mechanisms.

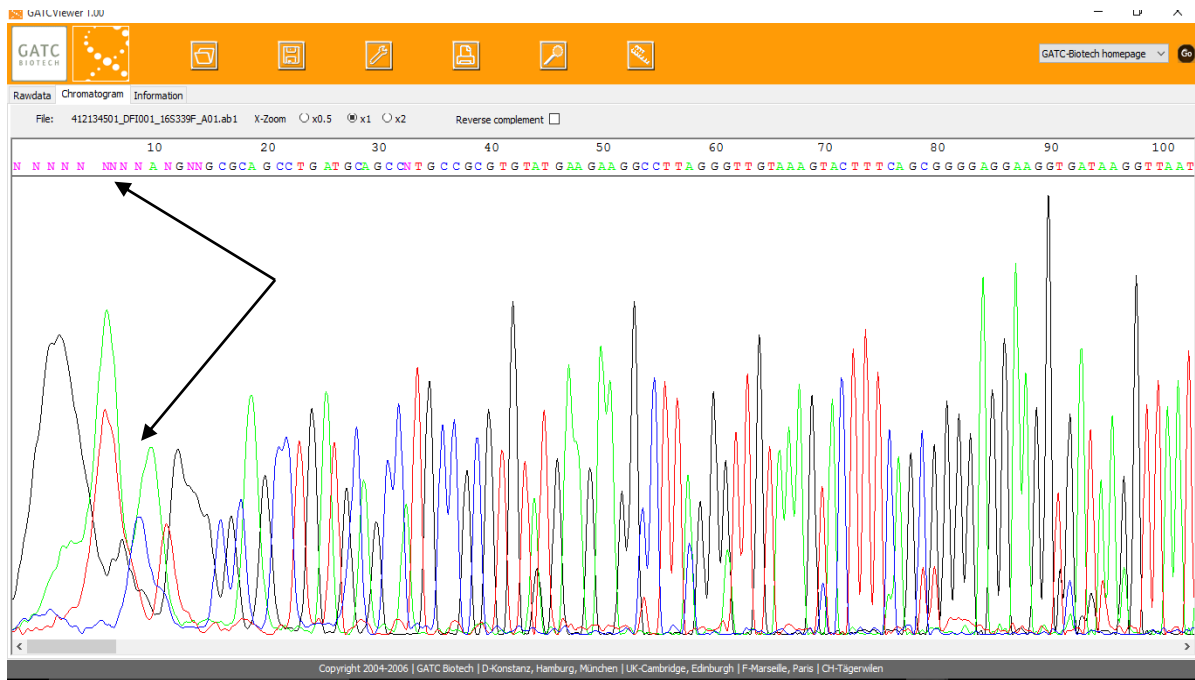


**Figure 3.5** Comparison of the susceptibility patterns of OPD *K. pneumoniae* and *P. mirabilis* strains and in-patient *K. pneumoniae* and *P. mirabilis* strains. The difference between the number of OPD *K. pneumoniae* and *P. mirabilis* strains sensitive to ceftazidime and levofloxacin and in-patient *K. pneumoniae* and *P. mirabilis* strains sensitive to ceftazidime and levofloxacin was statistically significant ( $*p < 0.05$ ). The difference between the number of OPD *K. pneumoniae* and *P. mirabilis* strains resistant to ceftazidime and levofloxacin and in-patient *K. pneumoniae* and *P. mirabilis* strains resistant to ceftazidime and levofloxacin was statistically significant ( $**p < 0.0001$ ).

### 3.5 Phylogenetic studies of clinical isolates from DFUs

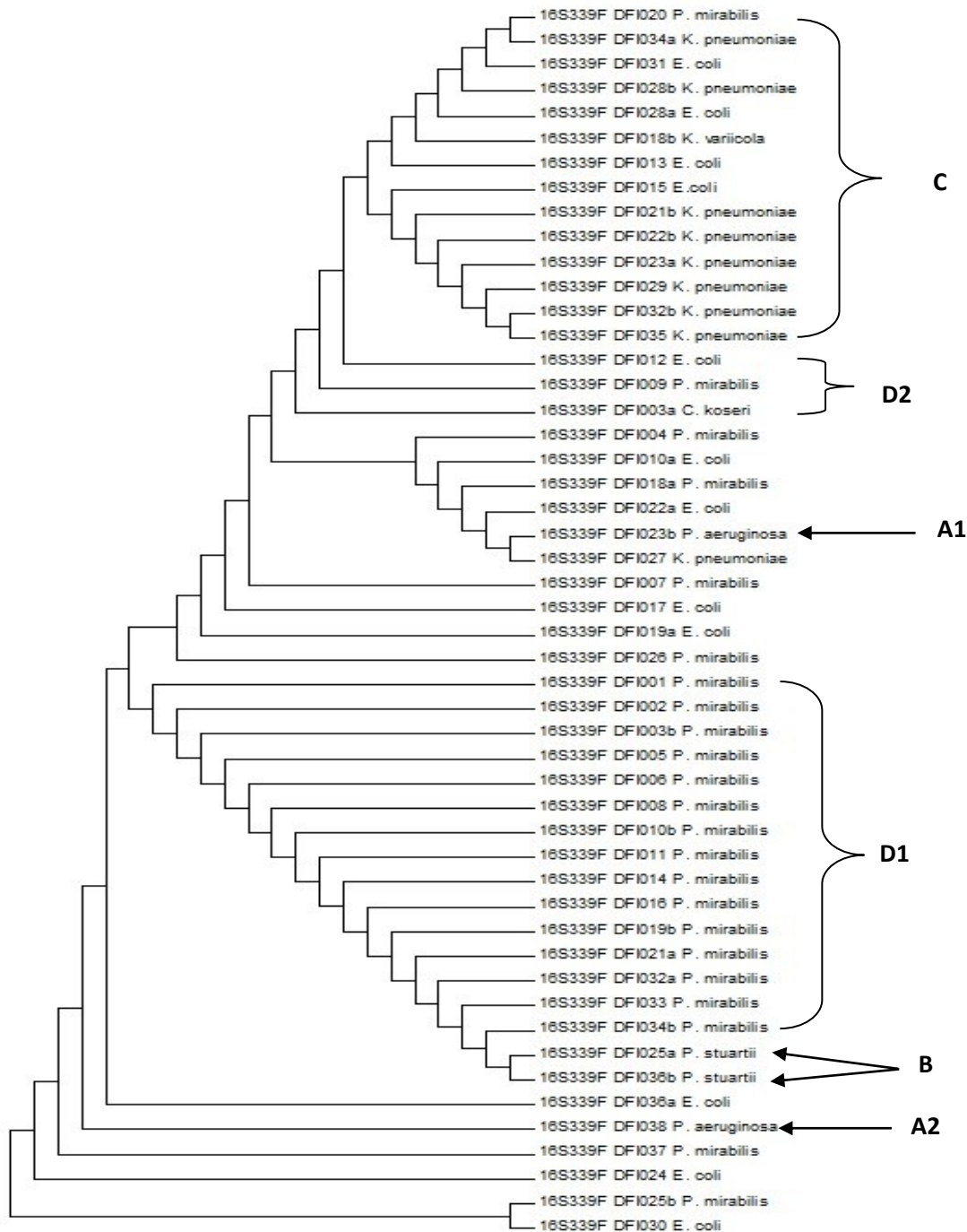
Before the construction of a tree of relatedness (phylogram), the chromatograms of all sequenced DNA were carefully observed for high quality reads and misreads using the GATCViewer 1.00 software (errors) (Figure 3.6). All sequences were retrieved from the chromatograms and saved as 'FASTA' (fas) files which can be used to submit sequences to either NCBI BLAST or MEGA6 for further analysis. Sequences with misreads (errors) were trimmed by removing all the misread base pairs prior to use. The construction of dendrogram was preceded by multiple sequences alignments. All 16S rRNA DNA sequences were

collected in a FASTA text format and analysed with MUSCLE alignment tool in MEGA6 to generate a multiple sequence alignment (Appendix C1).



**Figure 3.6** Chromatogram showing partial genome sequence of a 16S rRNA gene with base pairs. Arrowed peaks and base pairs (represented as – NNNNNNNN) indicate error readings (misread) of the first 15 base pairs of the sequence.

The tree of relatedness was constructed using the Neighbor–Joining method (Saitou and Nei, 1987). Phylogenetic analysis was subsequently carried out to study the ancestral relatedness of all 50 strains belonging to the Proteobacteria group. MEGA6 bioinformatics software was used to construct a Neighbor–Joining phylogenetic tree using 16S rRNA DNA gene sequences of all identified strains (Figure 3.7). The branches of the phylogenetic tree were divided into six regions namely; A (1 and 2), B, C, and D (1 and 2), based on either convergent or divergent relatedness of the taxa for analysis.



**Figure 3.7** Phylogenetic tree of 16S rRNA relatedness of clinical isolates using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 6.53474913 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2000) and are in the units of the number of base substitutions per site. The analysis involved 50 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 34 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

### 3.6 Discussion

The aim of this chapter was to study the epidemiology of diabetes and foot ulcers among patients attending the Diabetes Centre at the Komfo Anokye Teaching Hospital, Kumasi, Ghana. However due to scanty data retrieved from recruited participants, this chapter was dedicated to study the prevalence of DM and DFU among patients that attended KATH during the period of samples collection.

KATH is the second largest teaching hospital in Ghana with a current total bed capacity of 1200. Currently the average yearly OPD attendance at KATH has been estimated to be 54,000 including 20,239 children. The hospital is centrally located and caters for about half of the population of Ghana with its catchment area including the Ashanti, Brong Ahafo, Northern, Upper East and Upper West, Central, Eastern, Volta and some sections of the Western regions. This makes it ideal for such an important study. This is the first time the epidemiology of diabetes and DFU have been studied in this part of Ghana. The statistical data and demographics provided by the Ghana Statistics Services (GSS) make the Ashanti region a perfect location for studies that can make projections for national recommendations. Data on the epidemiology of the disease in Ghana and Africa are scanty. This study will therefore provide data and recommendations for future work in line with global forecast of the disease.

One of the aims for this study was to collate enough data to establish the current prevalence of DM and DFU among patients attending KATH, Kumasi, Ghana. The current prevalence rate established by the IDF was calculated based on comparative studies with other neighbouring developing countries due to lack of data in Ghana (Agoudavi *et al.*, 2012; Baldé *et al.*, 2007; Ministry of Health and Benin, 2007; Ministry of Health Botswana, 2007; Van Der Sande *et al.*, 1997). The diabetes prevalence rate of 22.8% among 54000 patients, estimated in the current study is the first to be established at KATH, the second largest teaching hospital in Ghana. However, a number of factors affected the high prevalence rate established by this study. One such factor is the population from which the participants were drawn. Unlike Amoah *et al.* (2002), who conducted a cross-sectional study based on random selection and recruitment of study participants from some communities based on recommendation by the Ghana Statistics Service, the participants for the current study, were



made up of a hospital-based controlled population whose diabetes statuses were already known. They included both old and newly diagnosed cases who regularly attended the diabetes centre at KATH. Another factor was the age range for enlisting participants onto both studies. Amoah *et al.* (2002) recruited participants who were  $\geq 25$  years whilst the current study recruited participants who were  $\leq 24$  to 60 years and over. This is because the current study was intended to establish a prevalence rate not only for the adult population but also for juveniles living with the disease. A third factor that might have contributed to the increased current prevalence rate was the fact that some of the DM participants recruited for the current study might have been referral cases from other part of the country including, Brong Ahafo, Eastern and Northern regions. Finally, participants' information collated for the current study was inconclusive which could have provided a more precise exclusion – inclusion criterion for eliminating biases in sampling. Some of these factors can be considered in the future to increase precision and provide a more accurate analysis of data.

DM was found to be about 2.5 times more prevalent in Ghanaian women than in men (72% and 28% respectively). Similar trends have been found in previous DM studies in Ghana (Amoah *et al.*, 2002; Darkwa, 2011; Owiredu *et al.*, 2008). In other studies, in Mauritius, India and China, DM was also found to be more prevalent in women than in men (Agrawal and Ebrahim, 2012; Magliano *et al.*, 2010; Yang *et al.*, 2010). However, the prevalence of the disease among men and women in developed countries like the UK and the USA are slightly higher in men than in women (Centers for Disease Control and Prevention, 2014; Centers for Disease Control and Prevention, 2015; Diabetes in the UK, 2012). It has also been found that a complex combination of genetic and environmental factors as well as social deprivation make DM about six times more common among people of South Asia descent and about three times more common in people of African and African-Caribbean lineage than Caucasians (Chen *et al.*, 2012; Diabetes in the UK, 2012). In the case of Ghana, it can be suggested that, the risk of developing DM is about 2.5 times higher in women than in men. The higher number of DM cases among Ghanaian women may be due to physical inactivity, obesity, unemployment, illiteracy and probably genetic predisposition (Chen *et al.*, 2012; Danquah *et al.*, 2012; Wild *et al.*, 2004). According to Danquah *et al.* (2012), illiteracy and other independently associated factors such as unemployment, crowded living conditions and living on the outskirts of town/cities or in villages with little or no access to information on

healthy living or adequate healthcare have been found to contribute to the increase in DM cases among Ghanaians.

Age-standardised prevalence of DM was found to be highest among the 45 – 59 age group representing 41.3% of cases (Table 3.4). Similarly, observations have previously been reported in Ghana (Amoah *et al.*, 2002; Darkwa, 2011; Owiredu *et al.*, 2008) and elsewhere in Africa (Evaristo-Neto *et al.*, 2010; Evaristo-Neto *et al.*, 2012; Kyari *et al.*, 2014; Nyenwe *et al.*, 2003; van der Sande *et al.*, 1997). The above-mentioned findings correlate with the global trend of the disease in developing countries as predicted by the WHO (King *et al.*, 1998; Zimmet *et al.*, 2001; International Diabetes Federation, 2013). Urbanisation, acculturation, obesity, aging and physical inactivity have been linked to increase in DM prevalence among this age group (Wild *et al.*, 2004). However, the age group associated with the highest prevalence in developed countries like the UK and USA is the 60 – 79 years' group (Centers for Disease Control and Prevention, 2014; Centers for Disease Control and Prevention, 2015; Diabetes in the UK, 2012). This is possibly due to difference in life expectancy between the developing and the developed countries. In Ghana, the estimated life expectancies at birth for males and females are from 60.2 – 62 and 63.4 – 64 respectively (Ghana Statistical Service, 2013; World Health Organisation, 2013). In developed countries, however, life expectancies for males and females are 79 and 81 respectively (Ediev, 2011; World Health Organisation, 2013).

On the other hand, DFU was more prevalent among diabetics who were 60 years and above (Table 3.5). In Ghana, most people belonging to this age group are peasant farmers or pensioners who live alone. They normally do not get help with the proper management of their diabetes hence the increase in DFU among them. Some of them also live in the country and have no access to health care or attend regular diabetes reviews at their designated health centres (Danquah *et al.*, 2012, Ministry of Health Ghana, 2013). Lack of properly managed community healthcare or wound nursing has also aggravated the situation (Ministry of Health Ghana, 2013).

The low prevalence (0.7%) of DFU established among diabetics recruited for this study is a significant achievement as far as global prevalence and predicted increases are concerned (Singh *et al.*, 2005; International Diabetes Federation, 2015). The current global prevalence ranges from 4 – 27% (Abbott *et al.*, 2002; Bakri *et al.*, 2012; Nathan *et al.*, 2008; Richard and

Schuldiner, 2008; Yazdanpanah *et al.*, 2015). It has been found that foot ulcers are less common in Asians and Afro-Caribbeans living with DM (Abbott *et al.*, 2005) hence the low prevalence in Ghanaian patients. Credit must also be given to the Nurse-in-charge and other staff at the Diabetes Centre, KATH who have embarked on a DFU sensitisation and awareness programme for the past 5 years called 'I want my leg back.' The programme has also contributed to the low DFU prevalence recorded especially in 2011 and 2012 among patients accessing the centre (Table 3.4). Like DM, DFU was more prevalent in diabetic women (61%) than in men (39%) unlike in developed countries where it is more common in men than in women (Boulton, 2015).

It has previously been mentioned in section 1.1.3 that DFU contributes to most hospital admissions among DM patients in Ghana with concomitant amputations and even death (Asumanu *et al.*, 2010). In this study, however, the number of OPD DFU cases was slightly higher (2.2%) than in-patient cases. Though the ratio of total OPD DFU cases to in-patient DFU cases over the 4 year-period of the study was approximately 1:1 (51.1%: 48.9%), their combined prevalence (0.7%) was significantly low with respect to global predictions and trend of the disease. There is a consensus that the more OPD DFU cases there are, the better the management and prognosis of the ulcers. On the other hand, the higher the number of in-patient DFU cases the higher the risks of amputation and/or death (Asumanu *et al.*, 2010; Setacci *et al.*, 2009). High risks of amputation and death among DFU patients in the nosocomial setting are mainly due to colonisation and infection of these chronic wounds by multidrug resistant bacteria that resist treatment and subsequently impair wound healing (World Health organisation, 2002; World Union of Wound Healing Societies, 2008).

It was also observed that, the number of in-patient and out-patient DFU samples with multispecies bacteria (maximum of 2 bacterial species per sample) were 37 and 26 pairs respectively. The recovery of more clinical isolates from in-patient DFU samples in the work presented here could be due to a number of factors including acquisition from other infected wounds in the ward (World Health Organisation, 2002). Others include from the infected hands of healthcare staff providing wound care, use of infected wound dressing equipment and from the microflora of surrounding skin or the healthcare environment (Cruse and Foord, 1980; Orrett *et al.*, 1998; World Health Organisation, 2002). Bacterial colonisation of DFUs in wound care units poses a threat to other patients with open wounds as they may also be

colonised and become reservoir hosts for the transmission of multidrug resistant pathogens (Dzidic and Bedeković, 2003; Mayon–White *et al.*, 1998). Lack of infection control measures can lead to widespread nosocomial infection and the dissemination of hospital–acquired antibiotic resistant bacteria into the community on the discharge of in–patients into the communities (Dzidic and Bedeković, 2003; Popovich *et al.*, 2008; Setacci *et al.*, 2009). The conglomeration of nosocomial and community–acquired resistant pathogens in the same ecological niche could provide the environment for the transfer of multidrug resistance genes and the widespread of multidrug resistant infections.

The work present here observed that the Proteobacteria group was the dominant bacteria (Table 3.6) constituting more than 90% of all clinical isolates identified. The Proteobacteria group has previously been found as major constituents of the microbiome of DFIs and are among the dominant bacteria in deep wounds (Gardner *et al.*, 2013; Malik *et al.*, 2013). For example, Gardner *et al.* (2013) were able to establish that the presence of Proteobacteria in deep wounds was associated with the increased duration of ulceration and the presence of a dominant species. They also established *Staphylococcus* species as the predominant species in superficial wounds because they have more oxygen to support their existence than in deep wounds which support the growth of mostly Proteobacteria and anaerobes. Out of the 38 DFUs categorised in Table 3.2, 31 (82%) were deep wounds (including gangrenous forefoot and abscess osteitis). Although all 38 wounds yielded 50 bacterial isolates belonging to the Proteobacteria group (Table 3.7), the relationship between wound depth, duration and their microbial diversity could not be established in the current work presented. This is due to the fact that no data was collated on the duration of ulcers. It can therefore be assumed that most of the remaining 318 DFU samples which were analysed by the staff at the Clinical Microbiology Department, KATH, Kumasi, Ghana were probably from deep wounds as they produced more than 90% bacterial species belonging to the Proteobacteria group. However, this assumption cannot be proven as there is no data evidence to support it.

The recovery of bacteria from the wounds sampled in the current study yielded growth corresponding to  $\geq 10^5$  CFU/mL. However, there was not enough evidence such as the characterisation of clinical factors like neuropathy, ulcer duration and glycaemic control to effectively establish the microbial bioburden of infected foot ulcers. The microbial burden, which is a function of microbial load, diversity and presence of functional equivalent

pathogroups, has been associated with impaired wound healing and amputation (Dowd *et al.*, 2008b; Gardner *et al.*, 2014; Spichler *et al.*, 2015). However, culture-based methods that mostly support the growth of non-fastidious bacteria have underestimated the microbial burden of DFIs (Bansal *et al.*, 2008; Gardner *et al.*, 2013; Lipsky *et al.*, 2013). As a result, molecular-based techniques such as 16S rRNA-based assays like DGGE, TGGE and pyrosequencing have been widely employed to establish the microbial bioburden of DFIs (Gardner *et al.*, 2013; Lavigne *et al.*, 2015; Salipante *et al.*, 2013). 16S rRNA-based assays such as 16S rRNA PCR amplification (Figure 3.3) and DNA sequencing (Figure 3.6) have also enabled the species-specific identification of the whole microbial diversity of DFIs using bioinformatics tools such as Neighbor-Joining, Fitch-Margoliash, Maximum-Parsimony, Maximum-Likelihood and Minimum-Evolution methods that allow the study of the evolutionary relationships between the microbiome of DFUs (Saitou and Imanishi, 1989). DFUs with high microbial burden lead to increased amputation, morbidity and mortality. Though this study did not fully investigate microbial burden of DFUs, it is probably sufficient to suggest that there was a relationship between microbes colonising DFUs and the presentation of clinical factors among DFU patients sampled in this study.

The distribution of bacteria in infected DFUs in a population, geographical location or treatment centres depends on a number of factors including the presence of dominant bacterial species in that region, the ecology of the wounds, host immune response and antibiotic selective pressure (Bansal *et al.*, 2008; Spichler *et al.*, 2015). The microbiological profiles of DFUs sampled in this study have been summarised in Tables 3.6 and 3.7. The microbiological profile identified included Gram-negative and Gram-positive strains belonging to two main phyla; Firmicutes (*Bacillus* and *Staphylococcus species.*) and Proteobacteria (all the Gram-negative strains). Similar findings were also observed in separate works carried out in Kuwait, Southwestern Nigeria and North and South India (Abdulrazak *et al.*, 2005; Ako-Nai *et al.*, 2006; Shankar *et al.*, 2005; Zubair *et al.*, 2011). Unlike the studies conducted in Ghana and other developing countries, a similar study conducted in Lisbon, Portugal, identified less than 30 % of Proteobacteria from DFUs (Mendes *et al.*, 2012). *Candida albicans* was the only eukaryotic organism identified in the Ghanaian study. The presence of yeast cells in DFIs has been associated with Wagner's grades 3, 4 and 5 DFUs with attendant osteomyelitis (Enderle *et al.*, 1999; Levin, 1998; Spichler *et al.*, 2015). Although most of the wounds sampled in the present study were

categorised as Wagner's wound grades 3, 4 and 5 wounds, *Candida* species was not determined in any of them.

The results from the current study suggest that the relative abundance of bacterial species like *P. aeruginosa*, *P. mirabilis*, *E. coli* and *K. pneumoniae* in the wounds of Ghanaian DFU patients can be attributed to the fact that they are ubiquitous and mostly found in environmental habitats, on the skin as normal flora, on solid surfaces and as nosocomial bacteria and their ability to perpetuate opportunistic infections (Grice and Segre, 2012; Misic *et al.*, 2014; Persson, 2010). The preponderance of members of the *Enterobacteriaceae* in the microbial ecology in Ghanaian DFU cases may be due to factors such as the indiscriminate use of antibiotics, which leads to selection of antibiotic resistance, as well as lack of proper maintenance of environmental and personal hygienic conditions. Similar conditions have been found to account for the high prevalence of Gram-negative pathogens in infections in developing countries like India (Malik *et al.*, 2013; Rawat *et al.*, 2012). In other studies, the dominant bacterial species isolated from DFIs were Gram-positive aerobes especially MRSA, coagulate negative *Staphylococcus* and other *Streptococcus* species (Citron *et al.*, 2007; Lipsky *et al.*, 2004). Their presence in DFUs either as single species or in a consortium (called FEPs) with other species has been associated with clinical factors such as microvascular disease, peripheral vascular disease neuropathy and hyperglycaemia, impaired wound healing and amputation (Gardner *et al.*, 2013; Malik *et al.*, 2013; Spichler *et al.*, 2015).

In this study, the susceptibility patterns of clinical isolates to some of the commonly used antibiotics for treating DFIs at KATH were evaluated (Table 3.8). Antibiotic resistance ranged from 27–77% (108 to 312 isolates) while sensitivity was from 2–65% (10 to 264 isolates). Though the difference between the number of antibiotic sensitive and resistant bacteria (Figure 3.4) was not statically significant ( $p = 0.25$ ) by Wilcoxon Signed Rank test, it is clinically a worrying scenario. The current study also compared the antibiotic susceptibility patterns of OPD and in-patient strains of *K. pneumoniae* and *P. mirabilis* (Figure 3.5) to 2 third generation cephalosporin and fluoroquinolone antibiotics (ceftazidime and levofloxacin respectively) commonly used in Ghana. Though antibiotic resistance among OPD *K. pneumoniae* and *P. mirabilis* strains was 5.9% more than in-patient strains (for both antibiotics tested), the difference between the total number of antibiotic sensitive and

resistant *K. pneumoniae* and *P. mirabilis* strains for both OPD and in-patient cases was statistically significant by two-way ANOVA;  $p < 0.0001$ . Also, there were more sensitive OPD and in-patient strains of *K. pneumoniae* and *P. mirabilis* (60%) than there were resistant strains (40%); a development which is good for the prognosis of DFUs infected with either *K. pneumoniae* or *P. mirabilis* or both.

The recovery of wound pathogens with increased antibiotic resistance to first line antibiotics such as ampicillin, gentamicin and ciprofloxacin has been widely reported (Papasian and Kragel, 1997; Spichler *et al.*, 2015). Ideally, the start of antibiotic regimen for the treatment of chronic infected wounds like DFIs should be empirically chosen, as microbiological analyses of clinically significant isolates and their antibiogram are important in confirming diagnosis and advising clinicians on the appropriate choice of antibiotic regimen (Bowler *et al.*, 2001). Though the *in vitro* antibiotic susceptibility of clinical isolates may not directly relate to the prevailing clinical conditions *in vivo*, it contributes to the successful management of chronic wounds (Bowler *et al.*, 2001).

In Ghana and some other developing countries, the treatment of DFIs is mostly by the use of broad-spectrum antibiotics and wound dressings before microbiological tests are requested or reports released. This practice of prolonged antibiotic treatment leads to selective pressure on the microbial flora of the wound and the subsequent acquisition of resistance by some strains (Boulton, 2015). The failure of antibiotic treatment in some wounds may be compounded by the route of delivery such as systemic administration, which may be impaired by ischaemia and distal sensory neuropathy (Jeffcoate and Harding, 2003). The presence of biofilms colonising DFUs have also been found to impair wound healing and have increased resistance to antibiotics and other antimicrobials and sustained host defence mechanisms than planktonic cells (James *et al.*, 2008; Lopez *et al.*, 2010; Wolcott *et al.*, 2008). A biofilm is a community of multicellular aggregates of bacteria or fungi (such as *Candida* species) enclosed in a self-produced extracellular matrix (James *et al.*, 2008). Bacteria in biofilms have been reported to acquire resistance for various antibiotics through the horizontal transfer of genetic elements such as plasmids and transposons (Martinez and Silley, 2010; Murray, 1991). The extracellular matrix also called extracellular polymeric substance has been described as a physical barrier that inhibits the penetration of antibiotics and large

antimicrobial proteins such as lysozyme and complement from the host immune system (Bjarnsholt *et al.*, 2005; Lewis, 2001).

The classification of the microbial diversity in the microbiome of infected DFUs is an important process in determining the microbiological profile of a given population or geographical area. The taxonomical relationships between members of the Gammaproteobacteria class of the phylum Proteobacteria have been well classified based on their 16S rRNA gene (also known as the small subunit (SSU) ribosomal RNA) and protein sequences (Chun *et al.*, 2007; Williams *et al.*, 2010). Other phylogenetic studies of this class of bacteria have reported that deep tree branches make it difficult to construct a large and well organised phylogenetic tree with good resolution using the 16S SSU ribosomal RNA (Gao *et al.*, 2009; Wu and Eisen, 2008). Additionally, the validity of the tree interpretation of some SSU rRNA-based phylogenies has been challenged by the idea of horizontal gene transfer which is thought to take place during bacterial biofilm formation (Baptiste *et al.*, 2005). However, in the event where multiple bacterial protein families are shared by bacterial consortia in a biofilm, phylogenetic signals that represent trends of vertical inheritance have been detected (Puigbo *et al.*, 2009). A phylogenetic signal has been defined as the tendency for microbial species that are related to resemble each other, more than they resemble species randomly drawn from a phylogenetic tree (Kamilar and Cooper, 2013). Though horizontal gene transfer is common among members of the Gammaproteobacteria class, the acquisition of a gene and its retention in the genome of the recipient depends on a strong positive selection for its functionality (Lerat *et al.*, 2003; Ochman *et al.*, 2000). However, the presence of a gene in the genome of the recipient homologous to the acquired gene makes the acquired gene an unlikely selection or redundant for its functions (Ochman *et al.*, 2000). Despite the above-mentioned limitations in the use of 16S rRNA as a phylogenetic marker, it is still a useful tool to study the ecological diversity of microbial populations (Wu and Eisen, 2008).

In the present study, sequence data from amplicons of the 16S rRNA of 50 clinical isolates were used to reconstruct a gammaproteobacterial phylogeny using the Neighbor-Joining method (Figure 3.7). To increase concordance and tree resolution, the tree of relatedness was focused on the genera of two major families; *Enterobacteriaceae* and *Pseudomonadaceae* of the Gammaproteobacteria class. The purpose of the SSU 16S rRNA reconstruction of the gammaproteobacterial phylogeny in this study was to demonstrate the microbial diversity of



the Ghanaian DFU ecology. The phylogeny of the 50 isolates demonstrates high degrees of convergent and divergent evolution among the strains. The *P. aeruginosa* strains labelled A1 and A2 in Figure 3.7 have divergently evolved with low phylogenetic signal between them. The possible interpretation to these divergently related *P. aeruginosa* strains maybe due to the lateral acquisition of genes selected for their functions during the evolutionary process leading to high degree of differences between them (Kamilar and Cooper, 2013; Ochman *et al.*, 2000). The 2 *P. stuartii* strains (labelled B) isolated from 2 different DFUs and from 2 different subjects, a male and a female were 100% related. The observation of an apparent absolute convergent evolution with high phylogenetic signal between the 2 *P. stuartii* strains may be interpreted as phylogenetic conservatism characterised by low levels of evolutionary activities, pleiotropy (the influence of two or more phenotypic traits by a single gene) low rates of biotic interactions such as competition with other microbes for new niches and survival, high levels of gene flow (Bradshaw, 1991; Losos, 2008; Wiens, 2004; Wiens and Graham, 2005; Wiens *et al.*, 2010). The tree region labelled C was made up of isolates with different degrees of convergent evolution from a common root (ancestor). This probably means they share similar traits such as pleiotropic genes that encodes for proteins responsible for moderate to high levels of antibiotic resistance or virulence (Losos, 2008). Also, the relative convergent relatedness between branches of the tree region labelled C (also members of the family *Enterobacteriaceae*) suggests that they can coexist in the same ecological niche with little or no competition (Wiens and Graham, 2005; Wiens *et al.*, 2010). The branches of the tree labelled D1 show gradual divergence to the root of the tree. Though they are all *P. mirabilis* strains, they might have acquired other genetic elements from the environment but have retained a few of their ancestral genes over the years (Kamilar and Cooper, 2013). The 3 different isolates in group D2; 012 *E. coli*, 018a *P. mirabilis* and 003a *C. koseri* demonstrate 100% divergent evolution (leading to instability of the root) but are linked to ancestors of the C and A1 subgroups. Though these 3 species are divergently related members of the *Enterobacteriaceae* family, their ancestors may probably be related with some common traits shared between them (Kamilar and Cooper, 2013).

The Gammaproteobacteria has been reported as one of the oldest bacterial taxonomic groups with one of the most ecologically diverse bacterial population including free-living species like *Azotobacter*, human pathogens like *E. coli*, *P. aeruginosa*, *Vibrio* and *Salmonella species*. as well as plant pathogens, such as *Xanthomonas species* (Lerat *et al.*, 2003). Recent

development in molecular biology and bioinformatics has made it possible to sequence and publish the genomic DNA of many prokaryotes to enable large scale construction of evolutionary events using whole genome sequences (Chun *et al.*, 2007; Gao *et al.*, 2009; Lerat *et al.*, 2003). The availability of large numbers of published genomic sequences and their taxonomic coverage will enable better examination of evolutionary relationships by pulling conserved sequences of large protein families together (Lerat *et al.*, 2003; Wu and Eisen, 2008). Multiprotein approach of phylogenetic analysis has been found to provide more robust trees with better resolutions and details of phylogenetic signals that may suggest obvious vertical or lateral gene inheritance among the different isolates (Williams *et al.*, 2010).

The presence of anaerobes such as *Clostridium perfringens*, *Fingoldia magna*, *Peptostreptococcus anaerobius*, *Prevotella bivia*, *Propionibacterium acnes* and *Fusobacterium nucleatum* has been found as one of the major contributors to impaired wound healing in DFU patients (Dharod, 2010; Dowd *et al.*, 2008a; Dowd *et al.*, 2008b; Gardner *et al.*, 2013; Jauhangeer, 2006). Dharod (2010) and Jauhangeer (2006) in their separate works established *P. bivia*, *P. acnes* and *F. magna* as important pathogens in DFUs in an Indian and Mauritian cohort studies respectively. Due to lack of a dedicated anaerobic culture system at the site of sample collection in Ghana, anaerobes were not isolated and analysed in the current study. The presence of and relative abundance of facultative anaerobes such *K. pneumoniae* and *P. mirabilis* in non-healing chronic wounds such as DFUs has been well documented (Bowler and Davies, 1999; Bowler *et al.*, 2001; Gardner *et al.*, 2014; Grice and Segre, 2012; Martin *et al.*, 2010). However, their contribution to the chronicity of wounds at the molecular level has not been well explored compared to other predominant Gram-negative wound pathogens such as *E. coli* and *P. aeruginosa* (Costerton *et al.*, 1999; Dowd *et al.*, 2008a; Kokare *et al.*, 2009; Lembre *et al.*, 2012; Ling *et al.*, 2015; Lopez *et al.*, 2010). In the current study, the relative abundance of the combined total of *Klebsiella* and *Proteus species*. was 36% representing more than one-third of all bacteria isolated in this study. Against the backdrop of the relative abundance of *Klebsiella* and *Proteus species* and their antibiotic susceptibility to CAZ and LEV (Table 3.7 and Figure 3.3) 2 in-patient strains, 028b *K. pneumoniae* and 005 *P. mirabilis* were subsequently selected as the main test strains for further investigations throughout the current study. In addition to their sensitivity to amikacin, ofloxacin, ciprofloxacin and tazobactam/piperacillin, 028b *K. pneumoniae* was also

completely sensitive to levofloxacin. On the other hand, 005 *P. mirabilis* was completely resistant to ceftazidime and intermediate resistant to levofloxacin in addition to being resistant to ofloxacin, ciprofloxacin and amikacin. It can therefore be suggested that, in line with the characteristics of *K. pneumoniae* and *P. mirabilis* mentioned in this chapter, such as their dominance in DFUs in the Ghanaian setting and their antibiotic resistance profiles, they can be employed as model strains just like previously used model strains, *E. coli* and *P. aeruginosa* (Lopez *et al.*, 2010), to study biofilm formation in DFUs and the mechanisms underlying bacterial resistance to antimicrobials.

## Chapter 4

The *in vitro* study and the effect of antimicrobial treatment on biofilm formation by diabetic foot isolates

## 4.1 Introduction

Diabetes-associated amputations have been found to be preceded by impaired wound healing, which is as a result of ischaemia, Charcot neuroarthropathy and foot ulcer with infection (Dowd *et al.*, 2008a; Boulton, 2015). Loss of protective covering of skin means that, all open wounds are susceptible to bacterial contamination and subsequent colonisation from both endogenous and exogenous sources (Percival *et al.*, 2015). Bacteria colonising chronic wounds like diabetic foot ulcers (DFUs) replicate and adhere to the wound beds without causing tissue damage (Dow *et al.*, 1999; Edwards and Harding, 2004). Therefore, colonisation alone does not necessarily lead to impaired wound healing (Edwards and Harding, 2004). However, critical colonisation, which is the transition between colonisation and invasive wound infection and characterised by lack of progress towards healing, may progress to critical infection or invasive wound infection. Invasive wound infection normally stimulates host immune response and host (Edwards and Harding, 2004). The inability of the host to sustain effective immune response against the replicating bacteria leads to critical infection which is characterised by more than  $10^5$  colony forming units (CFU) of bacteria per gram of host tissue with accompanied virulence and pathogenicity (Wysocki, 2002). Bacteria involved in critical colonisation of DFUs have been found to exist as polymicrobial communities called biofilms (James *et al.*, 2008; Wolcott *et al.*, 2008). Bacterial autoaggregation or coaggregation with other wound bacteria has been suggested as an important step that precedes biofilm formation (Hill *et al.*, 2010; Rickard *et al.*, 2003). Biofilm formation is a common trait by which bacteria and fungi adhere to solid surfaces (inert or living) and form structurally complex communities of multicellular aggregates enclosed in a self-produced extracellular matrix called extracellular polymeric substance (EPS). Percival *et al.* (2015) further noted that, in addition the virulence and pathogenicity of individual bacteria colonising DFUs, the presence of pathogenic biofilms plays a major role in impairing wound healing.

It has been estimated that biofilms account for about 80% of all infections (Centers for Disease Control and Prevention, 2001; Davies, 2003; Percival and Cutting, 2009) and 85% of lower-limb amputations in biofilm-infected wounds among diabetics (Adler *et al.*, 1999; Palumbo and Melton, 1985; Pecoraro *et al.*, 1991). Evidence of biofilm in wounds has been well documented through *in vitro* studies over the past 2 decades. Animal studies using

mouse models have demonstrated *Staphylococcus epidermidis* and *Staphylococcus aureus* biofilms in wounds from biopsies taken 3 – 60 hours after wound inoculation (Gallimore *et al.*, 1991; Akiyama *et al.*, 1996). Similar studies confirmed the presence of biofilms in experimental murine and porcine acute wounds (Rashid *et al.*, 2000; Serralta *et al.*, 2001). Serralta *et al.* (2001) made partial wounds on the back of experimental pigs which were inoculated with *Pseudomonas aeruginosa* and covered them with coverslips for 72 hours. Congo red stained coverslips of the 72–hours biofilms observed under the microscope revealed EPS matrix with embedded biofilms. In another study, Davies *et al.* (2008) observed *S. aureus* biofilms using light, scanning electron and epifluorescence microscopy from an *in vitro* porcine wound model. Laboratory mice and rats with induced diabetes have been employed to study impair wound healing when challenged with *P. aeruginosa* (PA01) (Zhao *et al.*, 2010). In human clinical studies, James *et al.* (2008) was able to demonstrate the presence of biofilms from tissue biopsies in chronic wounds using scanning electron microscopy.

Other *in vitro* methods used to study biofilms include the microtitre plate (MTP) assay and its derivatives to quantitatively estimate biofilm biomass formed by isolates recovered from wounds or infections (Merritt *et al.*, 2011; O’Toole *et al.*, 1999; O’Toole, 2011; Stepanovic *et al.*, 2001; Ceri *et al.*, 1999; Sepandj *et al.*, 2003; Bardouniotis *et al.*, 2003; Harrison *et al.*, 2006; Tomlin *et al.*, 2005). MTP–based assays enable the study of physiology and metabolic activities of biofilms as well as their quantitative analysis. For instance, the MTP–based assays have been used to study the effects of changes in environmental conditions such as nutrient concentration, temperature and pH on a growing biofilm (Cotton *et al.*, 2009; Moller *et al.*, 2008; Stapper *et al.*, 2004; Cochran *et al.*, 2000; Harjai *et al.*, 2005). A derivative of the MTP–based assay called the Minimum Biofilm Eradication Concentration (MBEC™) assay, a high–throughput technique, enables the study of antimicrobial efficacy on biofilms formed on pegs of a standardised density (Ceri *et al.*, 1999; Harrison *et al.*, 2006; Tomlin *et al.*, 2005).

In addition to MTP–based assays, agar–based methods that incorporate staining with dyes such as Congo red, ethidium bromide, Calcofluor white and Ziehl carbol–fuchsin have also been used to demonstrate components within a developed biofilm such as exopolysaccharides, EPS and bacterial cells (Davies *et al.*, 2008; Serralta *et al.*, 2001). The

study of the effects of environmental conditions on a growing or developed biofilm has enabled the determination of optimum conditions for the identification of bacterial strains responsible for biofilm-associated infections (Uhlich *et al.*, 2014). Jones *et al.* (2015) further suggested that pH determination of wounds could help in the identification and characterisation of non-healing wounds for effective treatment. This is because, a healthy skin has slightly acidic pH (4–6) while the presence of a wound disrupts the acidic environment of the skin resulting in a more neutral pH of about 7.4 (Schneider *et al.*, 2007). Two separate studies have suggested that the treatment of wounds with nonpermeable dressings and solutions that induce acidic pH lead to quicker healing time than when treated at pH of 7.4 (Schneider *et al.*, 2007; Wilson *et al.*, 1978). Therefore, the regular determination of wound pH can distinguish between healing and non-healing wounds.

In Ghana, the treatment for diabetic foot infections involves the administration of broad-spectrum antibiotics such as the quinolones (ciprofloxacin and levofloxacin), and third generation cephalosporins such as cefotaxime and ceftazidime. Other antibiotics used are metronidazole, clindamycin and aminoglycosides such as gentamicin and tobramycin (Bonham, 2001; Nelson *et al.*, 2006; O'Meara *et al.*, 2000). Other treatment options include topical applications of biocides such as povidone iodine 0.25–0.5 % hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) which has been reported to kill bacteria in wounds, induce connective tissue formation and promote wound closure (Goldenheim, 1993; Loo *et al.*, 2012). Honey has also been a traditional salve for wounds for centuries (Ankra-Badu, 1992; Molan, 2006; Zumla and Lulat, 1989). However, biofilm-infected wounds have been found to have increased resistance to antimicrobials and sustained host defence mechanisms than planktonic cells (Lopez *et al.*, 2010). For instance, the EPS acts as a diffusion barrier that inhibits the entry of antibiotics and other antimicrobials that target the bacterial cells embedded in the biofilm (Bjarnsholt *et al.*, 2005; Lewis, 2001; Mah and O'Toole, 2001). In addition to the structural protection provided by the EPS to the biofilm community, other mechanisms have been found to be responsible for the antimicrobial resistance in the biofilm community. Brown *et al.* (1988) and Walters *et al.* (2003) in their separate works found that growth rate and nutrient deprivation in a growing biofilm were regulatory modulators fundamental to antibiotic activity which affect the susceptibility of bacterial cells to antimicrobial agents. Biofilm communities respond to environmental stresses such as low oxygen and nutrient levels which lead to reduced metabolic activities and low growth rates. The resultant

physiological changes lead to changes in bacterial cell envelope composition such as fatty acids, extracellular proteins, polysaccharides and envelope proteins (Brown and Williams, 1985; Gilbert and Brown, 1978; Sutherland, 1982). Also, phenotypic changes of bacterial cells during biofilm formation leading to persister cell formation have been suggested to lead to antimicrobial resistance (Costerton *et al.*, 1999). Other resistance mechanisms include quorum sensing (Gilbert *et al.*, 2002; Haas *et al.*, 2002), efflux pump expression (Brooun *et al.*, 2000) and persister cells production (Keren *et al.*, 2004; Lewis, 2007).

The presence of resistant bacterial strains and persister cells in biofilms have been found to increase their susceptibility to antibiotics to about 100–1000 fold their minimum inhibitory concentrations (MICs) (Gilbert *et al.*, 2002; Lewis, 2001; Lewis, 2007). The inhibition and eradication of biofilms and persister cells are topics which have engaged debates in the microbiology community over the past decade (Conlon *et al.*, 2013; Gerdes and Ingmer, 2013; Hurst *et al.*, 2005; Keren *et al.*, 2004; Keren *et al.*, 2012; Lewis, 2001; Lewis, 2005; Lewis, 2007; Mah and O’Toole, 2001; Maisonneuve and Gerdes, 2014; Stewart, 2015; van Acker *et al.*, 2014). Throughout these debates, suggestions have been put forward for consideration in terms of finding a mechanism for biofilm disruption. Some of the approaches include the study of persister genes, bacterial metabolism and growth rate, bacterial response to changes in micro–environmental condition and effect of antimicrobial challenge on the growing biofilm (Lewis, 2010; van Acker *et al.*, 2014). With respect to persister eradication, Lewis (2010) suggested that the combination of a regular antibiotic with a compound capable of disabling the maintenance of persister cells could lead to the total disruption of biofilms. In line with the latter idea, Conlon *et al.* (2013) noted in their work that the combination of a cyclic peptide called acyldepsipeptide antibiotic (ADEP4) with a conventional antibiotic, rifampicin, completely eradicated *Staphylococcus aureus* biofilms *in vitro*. In their work, they introduced ADEP4 into the cytoplasm of *S. aureus* that activated and dysregulated the ubiquitous ClpP protease after binding to it. The ADEP4 – ClpP complex recognised and eliminated misfolded proteins in the growing biofilm thereby causing autolysis of the cells. This work suggests that there is hope in the finding a possible treatment regimen for treating biofilm–infected wounds.



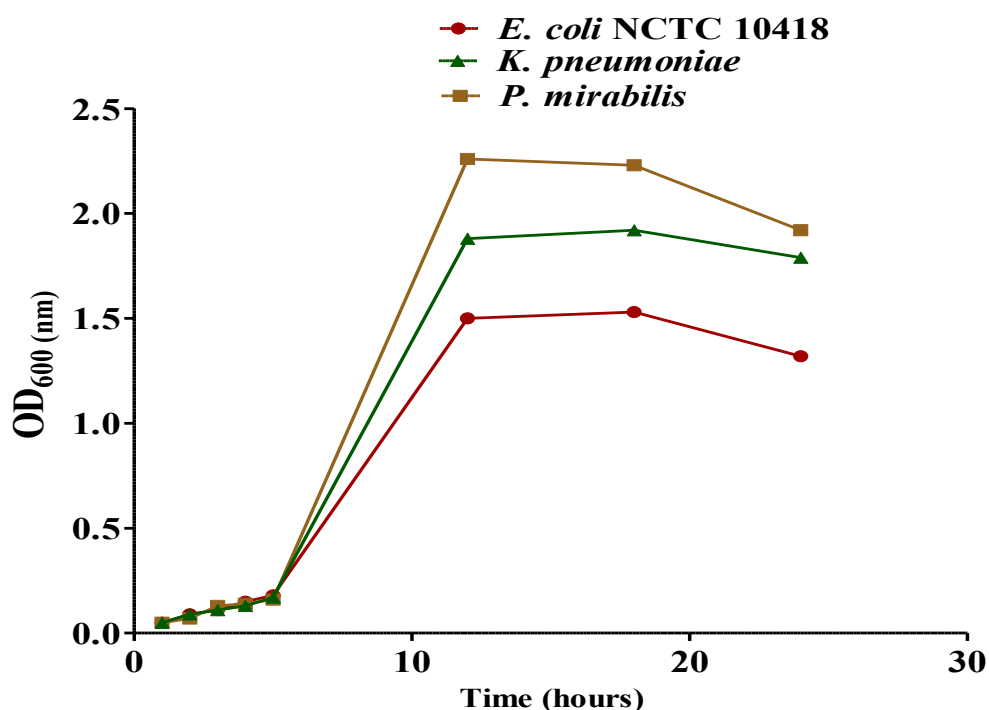
## 4.2 Aims and objectives

The role of biofilms in infections and especially in chronic wounds has been well documented as stated in section 4.1. The presence of biofilms in chronic wounds is of great concern when considering treatment strategies for implementation. Methods for estimation of biofilm formation and development *in vitro* of different clinical and environmental bacterial strains have also been well studied. However, two main issues are still outstanding as far as diagnosis of biofilm–infected wounds such a diabetic foot ulcers are concerned: the accurate quantitative estimation of biofilm in infections, and the presence of persister cells in biofilms (Hurst, 2005; Lewis, 2005; Lewis, 2007; Keren *et al.*, 2012). The aim of this chapter is to investigate biofilm formation among diabetic foot isolates using three different *in vitro* methods namely; the conventional 96–well microtitre plate assay, the MBEC™ assay and the *Quasi–Vivo*® system (Kirkstall, UK). This study will also consider the effect of antibiotics on *Klebsiella pneumoniae* and *Proteus mirabilis* biofilms which are the main representative test strains used in all assays (see Chapter 3, section 3.4 for more details). The following objectives will be considered in order to achieve the above aims;

- To determine the growth characteristics of diabetic foot isolates and their ability to coaggregate.
- To demonstrate *in vitro* biofilms formation and development and the production of the EPS.
- To study the effect of biofilm formation under different environmental conditions
- Isolation and characterisation of persister cells from planktonic and biofilm cultures of diabetic foot isolates.
- Determination of MIC, MBC and MBEC using both conventional MTP and MBEC™ high–throughput.
- Perform time–dependent killing assay on DFU isolates with some selected antimicrobials using the *Quasi–Vivo*® system.

### 4.3 Growth curve of clinical strains *K. pneumoniae* and *P. mirabilis*

The growth dynamics of test strains, *K. pneumoniae* and *P. mirabilis* together with *E. coli* NCTC 10418 reference strain, were monitored during the initial lag and log phases (0–5 and 5–12 hours respectively) and then the stationary and decline phases (12–18 and 18–24 hours respectively). Though the growth phases were only monitored for some selected time period, which were 1, 2, 3, 4, 5, 12, 18 and 24 hours, of the 24-hour period, it was observed that the test strains and the reference strain, *E. coli* NCTC 10418, have similar growth pattern (Figure 4.1). The estimation of bacterial biomass at the selected time points showed increases in biomass from  $5.7 \times 10^7$  to  $15.0 \times 10^8$  CFU/mL for *E. coli* NCTC 10418;  $6.7 \times 10^7$  to  $23.81 \times 10^8$  CFU/mL for *K. pneumoniae*; and from  $5.8 \times 10^7$  to  $22.41 \times 10^8$  CFU/mL for *P. mirabilis* with corresponding increases in OD<sub>600</sub>. The growth curve analysis of the test strains was helpful in the dilution of cultures and estimation of inoculum sizes for subsequent assays in this study.



**Figure 4.1** Growth curve for biofilm-producing test strains *K. pneumoniae* and *P. mirabilis* and reference strain *E. coli* NCTC 10418.

#### 4.4 Coaggregation assay

All 8 DFU isolates demonstrated their ability to form both intra- and inter-species aggregates. Using the visual scoring system based on visual examination of coaggregation with the eye, the degree of coaggregation was from 1+ to 3+. No isolate recorded a 0 or 4+ score either in autoaggregation or pairwise coaggregation assay after 2 hours of incubation. All isolates except *C. koseri*, recorded 3+ score at some point, either in an autoaggregation or with a coaggregation pair (Table 4.1). Selective coaggregation was not evident but 001 *P. mirabilis* and 015 *E. coli* formed more definite visible (2+) and large (3+) intra- and interspecies aggregates 2 hours post-incubation (Table 4.1). The autoaggregation and coaggregation score (intra- and interspecies respectively) for the remaining 6 isolate was from 1+ to 3+.

**Table 4–1** Coaggregation score\* after 2 hours of incubation for pairs of diabetic foot isolates

		1	2	3	4	5	6	7	8
	Isolate	003a	001	015	038	025a	021b	005	028b
1	<b>003a</b> <i>C. koseri</i>	<b>1+<sup>a</sup></b>	2+	2+	2+	1+	1+	2+	1+
2	<b>001</b> <i>P. mirabilis</i>		2+	2+	2+	2+	2+	3+	2+
3	<b>015</b> <i>E. coli</i>			2+	2+	2+	3+	2+	2+
4	<b>038</b> <i>P. aeruginosa</i>				2+	3+	3+	1+	1+
5	<b>025a</b> <i>P. stuartii</i>					3+	3+	2+	1+
6	<b>021b</b> <i>K. pneumoniae</i>						3+	1+	3+
7	<b>005</b> <i>P. mirabilis</i>							3+	2+
8	<b>028b</b> <i>K pneumoniae</i>								2+

\* – Coaggregation score (scale from 0 – 4) according to the visual scale described by Cisar *et al.* (1979) reported as; 0 – no visible aggregates; 1+ – small uniform aggregates; 2+ – definite visible aggregates without settling in suspension; 3+ – large aggregates settling easily leaving a turbid supernatant fluid; 4+ – large aggregates with clear supernatant which settles immediately after vortex.

<sup>a</sup> – autoaggregation controls (emboldened) made up of equal volumes of bacterial suspension and coaggregation buffer.

Coaggregation scores were also recorded 24 hours after incubation (Table 4.2) to determine whether initial scores recorded 2 hours post-incubation had increased or reduced with time. The visual score for 3 pairs of coaggregation pair 003a *C. koseri*/005 *P. mirabilis*, 001 *P. mirabilis*/015 *E. coli* and 025a *P. stuartii*/021b *K. pneumoniae* decreased from 2+ to 1+ and 3+ to 2+ respectively. Also, the 3 visual score for autoaggregation for 3 isolates, 025a *P. stuartii*; 021b *K. pneumoniae*; and 005 *P. mirabilis* decreased from 3+ to 2+. On the other hand, the visual score for 3 pairs of coaggregation pair 003a *C. koseri*/001 *P. mirabilis*, 001 *P. mirabilis*/038 *P. aeruginosa* and 015 *E. coli*/038 *P. aeruginosa* increased from 2+ to 3+ respectively. 003a *C. koseri*/025a *P. stuartii* coaggregation pair also recorded an increase from 1+ to 2+ (Table 4.2).

Quantitative coaggregation results are shown in the lower left half of Table 4.2 below the diagonal column. Quantitative autoaggregation/coaggregation results were calculated using the formula in section 2.3.4 in chapter 2 and expressed as a percentage. The percentage coaggregation estimated for the pairwise combination assays ranged from 8.3% to 53.4%. Autoaggregation/coaggregation percentages above 5% were regarded as positive (Shen *et al.*, 2005). In this study, none of the combination reactions was below 5%. The strongest combination reactions were between 038 *P. aeruginosa*–001 *P. mirabilis* (47.0%), 028b *K. pneumoniae*–021b *K. pneumoniae* (52.1%) and 038 *P. aeruginosa*–015 *E. coli* (53.4%). However, the corresponding visual score for the 3 strongest percentage coaggregation reactions was 3+ for each of the 3 pairs (Table 4.2).

**Table 4–2** Coaggregation visual score\* (after 24 hours) and percentage (%) coaggregation score (measured by a microtitre plate reader) described by Shen *et al.*, (2005) for pairs of diabetic foot isolates.

Isolate	003a	001	015	038	025a	021b	005	028b
<b>003a</b> <i>C. koseri</i>	1+ <sup>a</sup>	3+	2+	2+	2+	1+	1+	1+
<b>001</b> <i>P. mirabilis</i>	32.7	2+	1+	3+	2+	2+	3+	2+
<b>015</b> <i>E. coli</i>	23.6	15.0	2+	3+	2+	3+	2+	2+
<b>038</b> <i>P. aeruginosa</i>	21.6	47.0	53.4	2+	3+	3+	1+	1+
<b>025a</b> <i>P. stuartii</i>	15.9	23.8	21.4	31.3	2+	2+	2+	1+
<b>021b</b> <i>K. pneumoniae</i>	28.2	32.3	23.5	38.4	19.2	2+	1+	3+
<b>005</b> <i>P. mirabilis</i>	12.4	42.1	23.4	11.8	24.5	8.3	2+	2+
<b>028b</b> <i>K pneumoniae</i>	19.2	26.3	30.4	15.1	9.2	52.1	19.6	2+

\* – Coaggregation score scale description given in the footnote of Table 4.1. Highlighted scores show the difference between 2 and 24 hours readings. The scores in the diagonal grille indicate the autoaggregation visual score. The figures in the lower left half of the table (below the diagonal grille) represent the percentage coaggregation measured using a microtitre plate reader. The corresponding percentage (%) autoaggregation figures for the scores in the diagonal grille are as follows: **003a** – 11%, **001** – 21%, **015** – 30%, **038** – 48.5%, **025a** – 24.8%, **021b** – 44.8%, **005** – 48.0% and **028b** – 22.0%.

<sup>a</sup> – autoaggregation controls described in Table 4.1.

#### 4.5 Biofilm formation using the conventional microtitre plate assay (MTP)

Normal conditions for growth and development of biofilm was overnight incubation (18 – 24 hours) at a pH of 7.0 – 7.5, temperature of 37°C and under aerobic condition. All the 3 media (LB, TSB/TSB + 0.25% glucose and BHI) used to grow the representative clinical and control strains supported their growth with little or no difference in their optical densities and growth curve analysis (results not presented). As a result, LB agar and broth were selected for subsequent assays. Selection of clinical strains for the biofilm assays was based on their coaggregation profiles and their presence in polymicrobial wounds. All clinical strains were found to be biofilm producers (Table 4.3). The quantification of biofilm biomass was carried out according to the classification described by Pye *et al.* (2013). In this study, however, the classification of bacterial isolates as biofilm producers was slightly adjusted to take into

account changes made in the technique used; which included growth media, number of washing steps, type of solubiliser and concentration used. Clinical isolates and control strains were classified as either, weak, moderate or strong biofilm producers based on the definitions outlined in section 2.4.1 in chapter 2. Out of the 11 selected strains, 9 produced biofilms > 0.13 at OD<sub>570</sub> (Table 4.3).

**Table 4–3** Definition of biofilm production

Clinical Isolate	OD <sub>570nm</sub>	Weak Producers	Moderate	Strong
		(W) 0.05 ≤ OD < 0.13	Producers (M) 0.13 ≤ OD < 0.25	Producers (S) OD ≥ 0.25
003a <i>C. koseri</i>	0.103	W*		
015 <i>E. coli</i>	0.102	W		
018b <i>K. variicola</i>	0.199		M	
021a <i>P. mirabilis</i>	0.179		M	
021b <i>K. pneumoniae</i>	0.179		M	
025a <i>P. stuartii</i>	0.25			S
028b <i>K. pneumoniae</i>	0.286			S
005 <i>P. mirabilis</i>	0.312			S
038 <i>P. aeruginosa</i>	0.187		M	
NCTC <i>E. coli</i>	0.13		M	
NCTC <i>S. aureus</i>	0.140			S

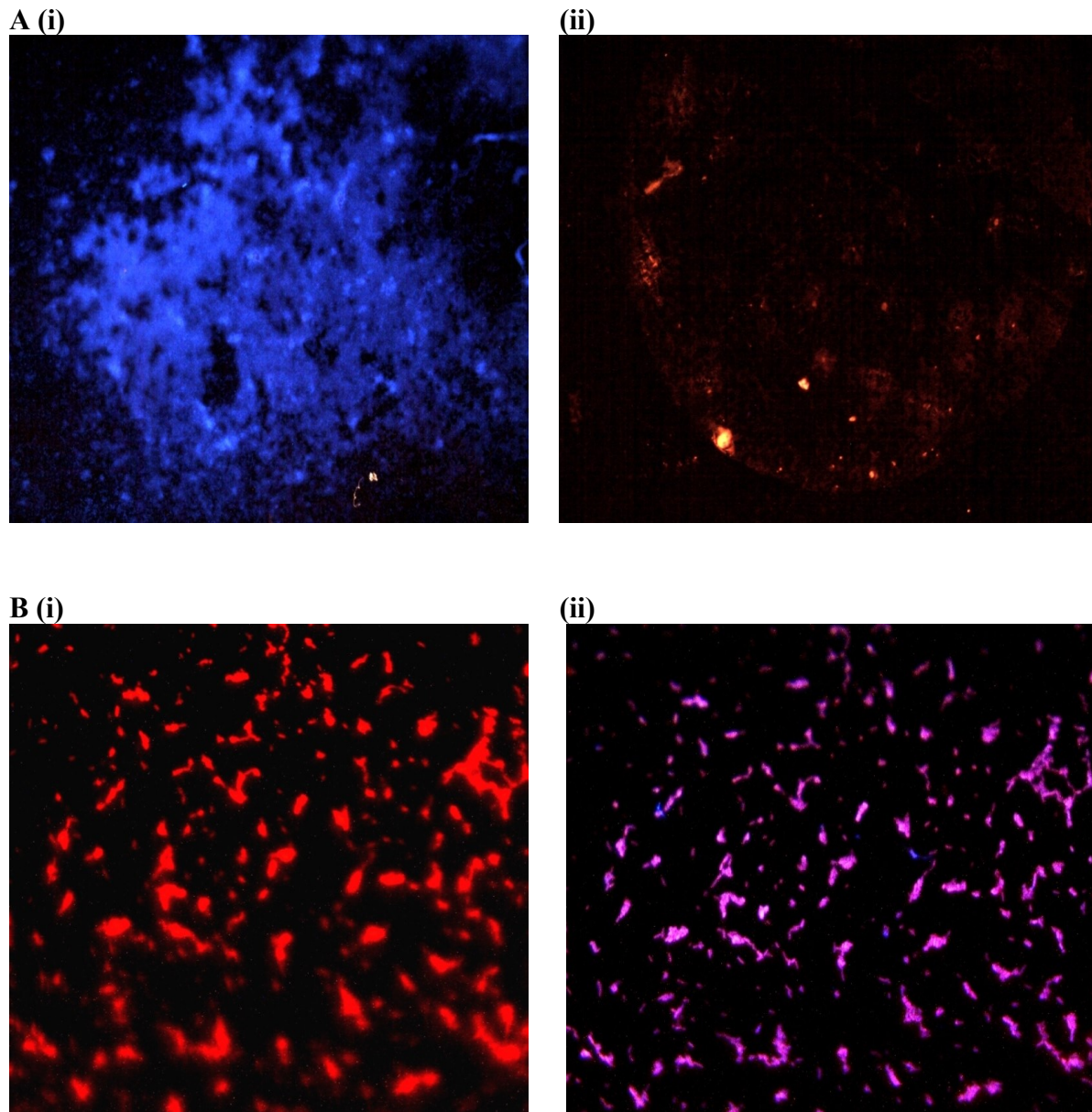
\* – Definition of biofilm producers; W – Weak producers; M – Moderate producers; and S – Strong producers

#### 4.5.1 EPS production during biofilm formation

The production of EPS by DFU isolates during biofilm formation was demonstrated and confirmed by 2 staining techniques. The stained slides were then visualised by epifluorescence microscopy that uses 2 filters to differentiate between stained polysaccharides and bacterial cells in the biofilm. In the first staining assay, the primary stain, Calcofluor White stained the polysaccharide matrix of the biofilm blue when viewed under the DAPI (4',6–diamino–2–phenyindole) light filter (Figure 4.3A (i)). The secondary stain,

ethidium bromide which inserts between the double strands of nucleic acids stains the bacterial cells which appear red under fluorescent light filters (Figure 4.3A (ii)).

The second confirmatory staining technique, Congo red and Ziehl carbol fuchsin dyes were used to stain the EPS. Under the DAPI filters, Congo red stains the polysaccharide matrix of the biofilm orange (Figure 4.3B (i)). The counterstain, Ziehl carbol fuchsin stained the bacterial cells purple under fluorescent light filters (Figure 4.3B (ii)).



**Figure 4.2** Epifluorescence biofilm images of: **(A) (i)** *P. mirabilis* EPS stained blue by Calcofluor white and **(ii)** nucleic acids stained red by ethidium bromide; and **(B) (i)** *K. pneumoniae* polysaccharide stained orange by Congo red and **(ii)** bacterial cells stained purple by Ziehl carbol fuchsin.



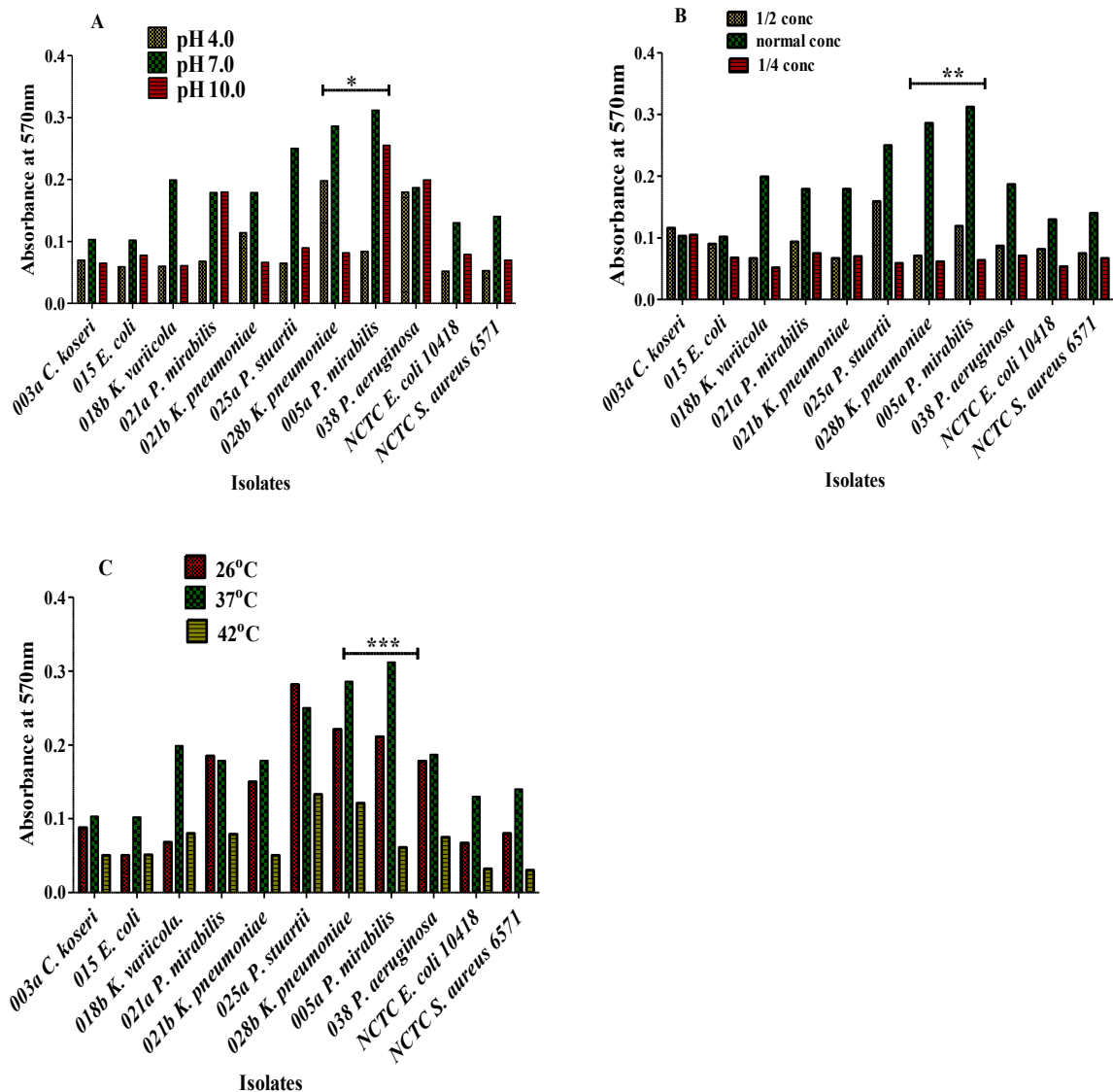
## 4.6 Effects of environmental conditions on biofilm formation

### 4.6.1 MTP assay for nutrient concentration, temperature and pH changes

The ability of selected clinical strains to form biofilms was determined under both normal conditions and other environmental conditions such as changes in temperature, pH and nutrient concentrations. To determine the statistical significance of the environmental changes made with respect to pH, temperature and nutrient concentration of the growth media and growth conditions, the set changes were compared to normal conditions (i.e., at pH 7, 37°C and full nutrient concentration). With the exception of 021a *P. mirabilis*, 028b *K. pneumoniae*, 005 *P. mirabilis* and 038 *P. aeruginosa* which showed significant growth ( $p = 0.0008$ ) at pH 4 or 10 (pH 4 and 10 for 038 *P. aeruginosa*), all other isolates showed reduction in biofilm formation when the pH was either 4 or 10 (Figure 4.4A). Biofilm reduction at both pH 4 and 10 was statistically significant for 018b *Klebsiella variicola* and 025a *P. stuartii* ( $p < 0.05$ ). At pH 4, biofilm reduction for 021a *P. mirabilis* was statistically significant ( $p < 0.05$ ).

The incubation temperature of 26°C did not affect biofilm formation in 6 out of the 11 strains tested (Figure 4.4 C). They included 003a *C. koseri*, 021a *P. mirabilis*, 021b *K. pneumoniae*, 025a *P. stuartii*, 028b *K. pneumoniae* and 038 *P. aeruginosa*. The difference between biofilm growth among these 6 strains at 26°C and 37°C was not significant ( $p > 0.05$ ). Biofilm formation in the remaining 4 isolates (015 *E. coli*, 018b *K. pneumoniae*, *E. coli* NCTC 10418 and *S. aureus* NCTC 6571) at 26°C was decreased by about half compared to normal growth temperature at 37°C ( $p < 0.05$ ). When incubation temperature was set at 42°C, biofilm formation by all 11 isolates was significantly decreased by half or less ( $p = 0.0001$ ) (Figure 4.4 C).

When nutrient concentrations in growth media were reduced by two-fold dilution, there was minimal effect on biofilm development in the case of 003a *C. koseri* and 015 *E. coli* (Figure 4.4 A). Biofilm development by 025a *P. stuartii* was decreased by one-third when nutrient concentrations were halved. In the cases of 028b *K. pneumoniae* and 005 *P. mirabilis*, the difference between biofilm biomass recovered at  $\frac{1}{2}$  and  $\frac{1}{4}$  concentrations compared to the control (normal nutrient concentration) was statistically significant ( $p < 0.0001$ ) (Figure 4.4B).



**Figure 4.3** Changes in the microenvironment during biofilm formation: (A) Effect of changes in pH; (B) Effect of reducing nutrient concentrations; and (C) Effect of changes in incubation temperature on biofilm. Differences between the range of environmental conditions for example, high and low pH, high and low temperature and normal and reduced nutrient concentration in growth media were determined using two-way ANOVA ( $*p < 0.05$ ;  $**p = 0.0001$ ; and  $***p < 0.0001$  respectively).

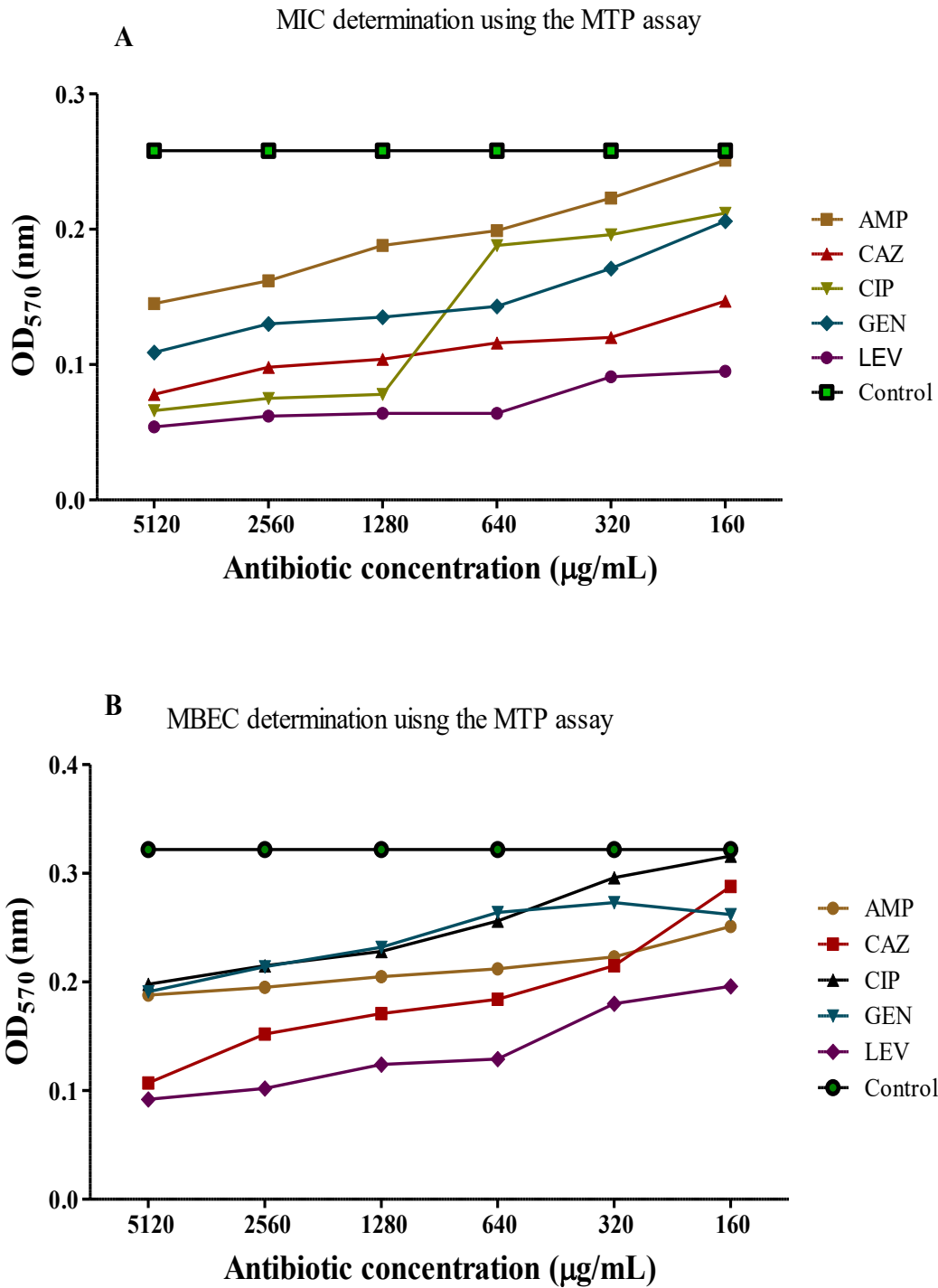
## 4.7 Biofilm inhibition and eradication assays

### 4.7.1 Determination of MIC, MBC and MBEC using the conventional MTP assay

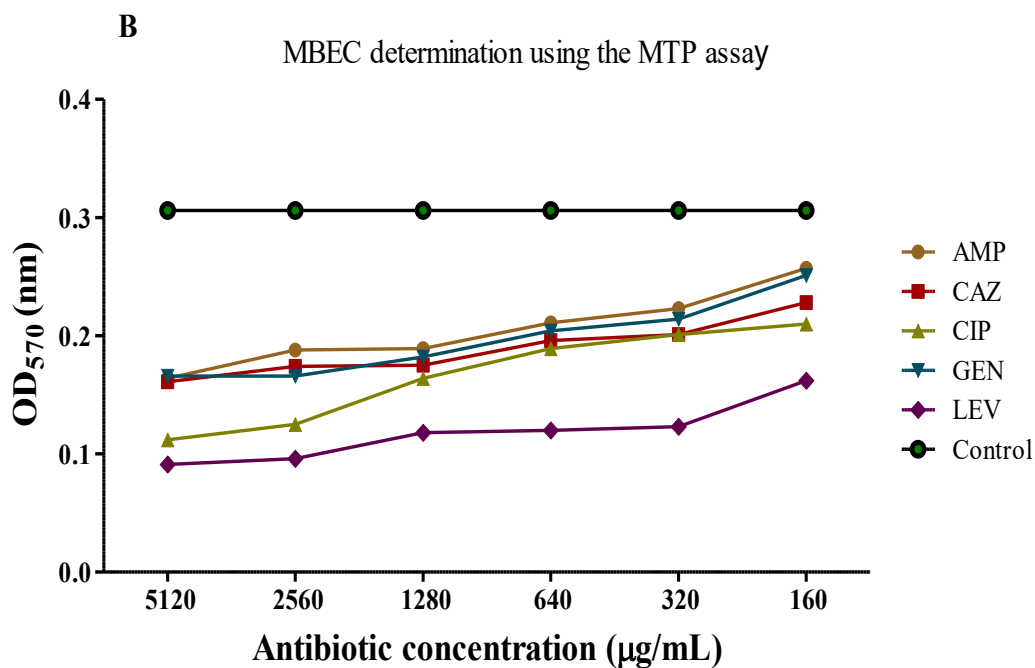
The efficacy of some selected antibiotics to inhibit and/or eradicate biofilm was tested on the *K. pneumoniae* (Figure 4.4A and B) and *P. mirabilis* (Figure 4.5A and B) respectively. Antibiotic selected for this assay included ampicillin (AMP), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (GEN) and levofloxacin (LEV). A working concentration of 5120 µg/mL for all antibiotics used was diluted in a two-fold dilution for the determination of MIC, MBC and MBEC (i.e., 5120, 2560, 1280, 640, 320, 160, 80, 40, 20 and 10 µg/mL). EUCAST antibiotic susceptibility testing breakpoints were used as guidelines for the interpretation of MIC data. For the purpose of this study, MIC and MBEC determinations using the conventional MTP biofilm assay were defined by percentage inhibition and eradication (respectively) of bacteria with  $OD_{570} \leq 0.05$  representing complete inhibition or eradication of biofilm.

The efficacy of antibiotics in inhibiting *K. pneumoniae* and *P. mirabilis* biofilms ranged from 44% to 75%; and from 59% to 71% respectively. Levofloxacin was the most effective antibiotic (75%) in significantly inhibiting *K. pneumoniae* biofilm formation ( $p = 0.0001$ ) at an MIC  $\geq 640$  µg/mL (Figure 4.4 A, Table 4.4). Ceftazidime and ciprofloxacin also recorded efficacies of 70% at MIC  $\geq 5120$  and  $\geq 1280$  µg/mL respectively (Table 4.4). Ampicillin and gentamicin were the least effective with MIC  $> 5120$  µg/mL against both *K. pneumoniae* and *P. mirabilis* (Tables 4.4 and 4.5).

There was no observable growth on LB agar plates inoculated with samples from the selected wells that showed efficacy  $\geq 70\%$ . With the exception of LEV, the MBC values for all the other antibiotics against *K. pneumoniae* and *P. mirabilis* were  $> 5120$  µg/mL. The MBC values for LEV against *K. pneumoniae* and *P. mirabilis* were 640 and 1280 µg/mL respectively. The MBEC values determined for all 5 antibiotics against both test strains were  $> 5120$  µg/mL (Table 4.5). However, the efficacy of LEV in eradicating 71% of *K. pneumoniae* and 70% of *P. mirabilis* biofilms was found to be statistically significant ( $p < 0.0001$ ) as shown in Figures 4.4B and 4.5B.



**Figure 4.4** Determination of MIC and MBEC of 5 selected antibiotics (ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin) against; (A) *K. pneumoniae* planktonic cells ( $p = 0.0001$ ) and (B) *K. pneumoniae* biofilms ( $p < 0.0001$ ).  $P$  values were determined by two – way ANOVA.



**Figure 4.5** Determination of MIC and MBEC of 5 selected antibiotics (ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin) against (A) *P. mirabilis* planktonic cells ( $p = 0.0002$ ) and (B) *P. mirabilis* biofilms ( $p < 0.0001$ ).  $P$  values were determined by two – way ANOVA.

**Table 4–4** Antibiotic efficacy of 5 antibiotics in inhibiting *K. pneumoniae* and *P. mirabilis* biofilms

Antibiotic (µg/mL) / Percentage inhibition (PI) (%)					
Bacterial strain	AMP*/PI	CAZ/PI	CIP/PI	GEN/PI	LEV/PI
<i>K. pneumoniae</i>	> 5120 (44)	≥ 5120 (70)	≥ 1280 (70)	> 5120 (58)	≥ 640 (75)
<i>P. mirabilis</i>	> 5120 (60)	> 640 (65)	> 1280 (65)	> 5120 (59)	≥ 1280 (71)

\* – Antibiotic: AMP – Ampicillin; CAZ – Ceftazidime; CIP – Ciprofloxacin; GEN – Gentamicin; LEV – Levofloxacin. Percentage inhibition/reduction (PI/PR) values were calculated as outlined in section 2.4.3.7 in chapter 2.

**Table 4–5** Antibiotic efficacy of 5 antibiotics in eradicating *K. pneumoniae* and *P. mirabilis* biofilms

Antibiotic (µg/mL) / Percentage reduction (PR) (%)					
Bacterial strain	AMP*/PR	CAZ/PR	CIP/PR	GEN/PR	LEV/PR
<i>K. pneumoniae</i>	> 5120 (42)	> 5120 (67)	> 5120 (39)	> 5120 (41)	≥ 5120 (71)
<i>P. mirabilis</i>	> 5120 (46)	> 5120 (47)	> 5120 (63)	> 5120 (46)	≥ 5120 (70)

\* – Antibiotic: See footnote of Table 4.4 for details.

The individual antibiotic efficacy of CAZ and LEV were tested against mixed biofilm of *K. pneumoniae* and *P. mirabilis* to determine their MIC and MBEC values as illustrated in Tables 4.6 and 4.7 respectively. The MIC and MBC of CAZ against mixed *K. pneumoniae* and *P. mirabilis* biofilm was > 320 µg/mL. However, the MIC and MBC of levofloxacin was estimated as ≥160 µg/mL and inhibited *K. pneumoniae* and *P. mirabilis* mixed biofilm by 83%. The MIC assay plates were incubated up to 72 hours. There was however no significant

difference between the readings recorded over the 3 days' period. This means that both antibiotics have sustained inhibitory effects on biofilm formation up to 72 hours.

**Table 4–6** Antibiotic efficacy of CAZ and LEV in the inhibition of *K. pneumoniae* and *P. mirabilis* mixed biofilm.

Antibiotic concentration (µg/mL) /Control	Absorbance at 570nm	Mean antibiotic activity (%)	Mean residual biofilm biomass (%)
Ceftazidime (320)	0.198	56	44
Levofloxacin (160)	0.077	83	17
Control (33% acetic acid) *	0	0	0

\* – Acetic acid (33%) was used as a control to blank the microtitre plate reader during all readings. OD<sub>570</sub> of positive mixed biofilm was 0.452. Mean antibiotic activity and mean residual biofilm biomass were determined using the formulae given in section 2.4.3.7 in chapter 2.

The individual antibiotic efficacy of CAZ and LEV to eradicate half or more of the mixed *K. pneumoniae* and *P. mirabilis* biofilm was found to be 320 and 160 µg/mL respectively (Table 4.7). It was however observed that CAZ (320 µg/mL) had almost the same effect in inhibiting and eradicating *K. pneumoniae* and *P. mirabilis* mixed biofilm. In the case of LEV, it was found to be about 24% less active in the eradication of *K. pneumoniae* and *P. mirabilis* mixed biofilm but at half the efficacy of CAZ.

**Table 4–7** Effect of CAZ and LEV in the eradication of *K. pneumoniae* and *P. mirabilis* mixed biofilm

Antibiotic concentration (µg/mL) /Control	Absorbance at 570nm	Mean antibiotic activity (%)	Mean residual biofilm biomass (%)
Ceftazidime (320)	0.146	57	43
Levofloxacin (160)	0.139	59	41
Control (33% acetic acid)	0	0	0

\* – Acetic acid (33%): OD<sub>570</sub> of positive mixed biofilm was 0.341. See footnote of Table 4.6 for further details.

The combined antibiotic efficacy of CAZ and LEV were also tested against mixed *K. pneumoniae* and *P. mirabilis* biofilm in 2 separate assays to determine their combined inhibitory and eradication concentrations respectively (Tables 4.8. and 4.9). It was observed that the combined effect of CAZ and LEV at 160 µg/mL inhibited mixed *K. pneumoniae* and *P. mirabilis* biofilm by 76% (Table 4.8). The combined MIC and MBC were found to be > 160 µg/mL.

**Table 4–8** Combined effects of CAZ and LEV in inhibiting mixed biofilm of *K. pneumoniae* and *P. mirabilis*

Antibiotic concentration (µg/mL) /Control	Absorbance at 570nm	Mean antibiotic activity (%)	Mean Residual biofilm biomass (%)
Ceftazidime and Levofloxacin (160)	0.098	76	24
Control (33% acetic acid)	0	0	0

OD<sub>570</sub> of mixed biofilm was 0.409. There was no significant difference between assays incubated for 24 hours and 72 hours. See footnote of Table 4.6 for further details.

It was also observed that the combined efficacy of CAZ and LEV at 640 µg/mL, tested against *K. pneumoniae* and *P. mirabilis* mixed biofilm eradicated only 39% of biofilm formed (Table 4.9). The combined MIC and MBC were found to be > 1280 µg/mL. Interestingly, it was observed that the individual antibiotic efficacy of CAZ (320 µg/mL) and LEV (160 µg/mL) were more effective (Table 4.7) in eradicating *K. pneumoniae* and *P. mirabilis* mixed biofilm than their combined efficacy (640 µg/mL) (Table 4.9). Similarly, LEV (160 µg/mL) was more effective in inhibiting *K. pneumoniae* and *P. mirabilis* mixed biofilm (Table 4.6) than when in combination with CAZ at the same concentration (Table 4.8).



**Table 4–9** Combined effects of CAZ and LEV in eradicating mixed biofilm of *K. pneumoniae* and *P. mirabilis*

Antibiotic concentration (µg/mL) /Control	Absorbance at 570nm	Mean antibiotic activity (%)	Mean residual biofilm biomass (%)
Ceftazidime and Levofloxacin (640)	0.239	39	61
Control (33% acetic acid)	0	0	0

OD<sub>570</sub> of mixed biofilm was 0.394. There was no significant difference between assays incubated for 24 hours and 72 hours. See footnote of Table 4.6 for further details.

When the individual efficacy of CAZ and LEV were assessed on *K. pneumoniae* and *P. mirabilis* mixed biofilms, LEV at 160 µg/mL, significantly inhibited *K. pneumoniae* and *P. mirabilis* mixed biofilms 20% more than as individual strains (Table 4.6). On the other hand, there was no significant difference between the inhibition of *K. pneumoniae* and *P. mirabilis* individual biofilms (53 and 63% respectively) and *K. pneumoniae* and *P. mirabilis* mixed biofilm (56%) by CAZ at 320 µg/mL. Again, the MBEC of CAZ (320 µg/mL) needed to eradicate more than 50% of *K. pneumoniae* and *P. mirabilis* mixed biofilm (Table 4.7) was 8 and 16 times (respectively) less than that needed by CAZ to eradicate 50% or more of *K. pneumoniae* and *P. mirabilis* individual biofilms. In the case of LEV, the MBEC (160 µg/mL) needed to eradicate 50% or more of *K. pneumoniae* and *P. mirabilis* mixed biofilm was 2 and 4 times less than that needed by LEV to eradicate 50% or more of *K. pneumoniae* and *P. mirabilis* individual biofilms.

Interestingly, the combined efficacy of CAZ and LEV at 160 µg/mL each (Table 4.8), inhibited *K. pneumoniae* and *P. mirabilis* mixed biofilm by 76%. LEV at the same concentration was 7% more effective whiles CAZ was less effective even at twice (320 µg/mL) the concentration of LEV (Table 4.6). Also, the combined effects of CAZ and LEV (640 µg/mL each) could only eradicate 39% of the mixed biofilm (Table 4.9). The individual efficacies of CAZ (320 µg/mL) and LEV (160 µg/mL) were 18 and 20% more effective at MBEC 2 and 4 times lower respectively.

#### 4.7.2 Determination of MIC, MBC and MBEC using the MBEC™ HTP assay

In the MBEC™ assay, 2 of the 5 antibiotics (ceftazidime and levofloxacin) previously used in the conventional MTP assay (section 4.5.1) above were selected and used to challenge *K. pneumoniae* and *P. mirabilis* in a series of assays to determine their MIC, MBC and MBEC values (Table 4.11). CAZ and LEV were selected because they were more effective against *K. pneumoniae* and *P. mirabilis* biofilms than AMP, CIP and GEN. The results obtained in MBEC™ assay together with that obtained using the conventional 96–well MTP assay were compared. For the MBEC™ assay, MIC and MBEC determinations were defined as percentage inhibition and eradication/removal (respectively) of biofilm with OD<sub>650</sub> < 0.1 representing complete inhibition or eradication of biofilm after appropriate incubation period (Allan *et al.*, 2011). The definition of MBC (i.e., the lowest concentration of antibiotic that can prevent the growth of bacteria after subculture onto media free from antibiotic) was maintained as described in section 2.4.2.2 (Andrews, 2001). However, MIC, MBC and MBEC values of at least 3 replicate assays were determined following the reading of absorbance at OD<sub>650</sub> using a microtitre plate reader.

**Table 4–10** Biofilm growth check (BGC) for bacterial strains

Bacterial strains	Log <sub>10</sub> CFU/mL	Log <sub>10</sub> CFU/mL	Log <sub>10</sub> CFU/mL	Average Mean
	Replicate 1 (well F12)	Replicate 2 (well G12)	Replicate 3 (well H12)	values (Log <sub>10</sub> CFU/peg)
<i>K. pneumoniae</i>	7.78	7.0	7.0	7.90
<i>P. mirabilis</i>	8.15	7.60	8.08	8.48

Table 4.10 represents biofilm growth (positive) control estimated from 3 replicates (1 peg per plate). The average CFU/peg was calculated as  $7.94 \times 10^7$  CFU/peg for *K. pneumoniae* and  $3.02 \times 10^8$  CFU/peg for *P. mirabilis*. The difference between biofilm growths on replicate pegs for both *K. pneumoniae* and *P. mirabilis* was found to be statistically insignificant (two–way ANOVA;  $p = 0.224$ ). This also means that, the pegs challenged during the MIC, MBC and MBEC assays formed equivalent biofilms across the rows.

The MIC, MBC and MBEC values of CAZ in inhibiting and/or eradicating *K. pneumoniae* and *P. mirabilis* biofilms were  $\geq 5120$   $\mu\text{g/mL}$  (Table 4.11). However, the MIC of CAZ in inhibiting *P. mirabilis* biofilm was 640  $\mu\text{g/mL}$ , 8 times lower than that against *K. pneumoniae*. Levofloxacin at MIC 40  $\mu\text{g/mL}$  completely inhibited *K. pneumoniae* and *P. mirabilis* biofilm formation (Table 4.11). However, the MBC of LEV (2560  $\mu\text{g/mL}$ ) needed to completely prevent the growth of *P. mirabilis* was 8 times more than that (320  $\mu\text{g/mL}$ ) needed for *K. pneumoniae*.

**Table 4–11** Residual *K. pneumoniae* and *P. mirabilis* biofilm ( $\text{Log}_{10}$  reduction) estimation after antibiotic challenge. MIC, MBC and MBEC values were expressed as percentage (%) of their  $\text{Log}_{10}$  reduction (Mean CFU/peg) values.

Bacterial strain	Antibiotic	Log <sub>10</sub> reduction		
		MIC ( $\mu\text{g/mL}$ )/ PI <sup>a</sup> (%)	MBC ( $\mu\text{g/mL}$ )/ PC (%)	MBEC ( $\mu\text{g/mL}$ )/ PE (%)
<i>K. pneumoniae</i>	Ceftazidime	$\geq 5120$ (88)	$\geq 5120$ (83)	$> 5120$ (37)
	Levofloxacin	40 (95)	320 (96)	$\geq 5120$ (84)
<i>P. mirabilis</i>	Ceftazidime	640 (80)	5120 (91)	$> 5120$ (60)
	Levofloxacin	40 (90)	2560 (84)	$> 5120$ (77)

<sup>a</sup> – Definition of percentage  $\text{Log}_{10}$  reduction: PI – percentage inhibition; PC – percentage clearance; and PE – percentage eradication. Percentage  $\text{Log}_{10}$  reduction values were calculated as outlined in section 2.4.3.3 in chapter 2.

It should be mentioned that the concentrations of antibiotics used to achieve the MIC, MBC or MBEC in the inhibition and/or eradication of *K. pneumoniae* and *P. mirabilis* biofilms using the MBEC<sup>TM</sup> assay were above the required therapeutic range for treatment of clinical conditions. The antibiotic therapeutic range for treatment of *K. pneumoniae* and *P. mirabilis* using CAZ and LEV are given as 0.125–4.0 mg/L and 0.25–4.0 mg/L respectively (EUCAST, 2014).

In the MBEC<sup>TM</sup> assay,  $\text{Log}_{10}$  reduction (residual biofilm) estimations (Table 4.11) were made after the MIC, MBC and MBEC determinations of CAZ and LEV. The  $\text{Log}_{10}$  reduction estimates provide data on the extent of inhibition and/or eradication of *K. pneumoniae* and *P.*

*mirabilis* biofilms after antibiotic treatment. The MIC of LEV that completely inhibited individual *K. pneumoniae* and *P. mirabilis* biofilms was found to be 40 µg/mL; 10 times more than the reference MIC. However, neither CAZ nor LEV could completely eradicate biofilm at concentrations > 5120 µg/mL. The MBCs of LEV and CAZ that completely prevented the growth of *K. pneumoniae* and *P. mirabilis* determined at OD<sub>650</sub> and by viable cell count on agar plate were 320 and 5120 µg/mL respectively.

**Table 4–12** Comparing MIC and MBEC determinations using the Conventional MTP and MBEC™ assays

Bacterial strain	Antibiotic	Conventional MTP assay			
		MIC (µg/mL)	PI* (%)	MBEC (µg/mL)	PR* (%)
<i>K. pneumoniae</i>	CAZ	≥ 5120	70	> 5120	67
	LEV	≥ 640	75	≥ 5120	71
<i>P. mirabilis</i>	CAZ	> 640	65	> 5120	47
	LEV	≥ 1280	71	≥ 5120	70
		MBEC™ assay			
<i>K. pneumoniae</i>	CAZ	≥ 5120	88	> 5120	37
	LEV	40	95	≥ 5120	84
<i>P. mirabilis</i>	CAZ	640	80	> 5120	60
	LEV	40	90	> 5120	77

\* – Definition of percentage Log<sub>10</sub> reduction: PI (%) – percentage inhibition; PR (%) – percentage reduction. Percentage Log<sub>10</sub> reduction values were calculated as outlined in section 2.4.3.3 in chapter 2.

In Table 4.12, it was observed that the MIC values of LEV for *K. pneumoniae* and *P. mirabilis* using the MBEC™ assay recorded higher percentage inhibition values with concentrations 16 – 32 times lower than with the MTP assay. Though the MBEC of CAZ and LEV were ≥ 5120 µg/mL for both MTP and MBEC™ assays, percentage eradication of *K. pneumoniae* and *P. mirabilis* biofilms obtained from the MBEC assay were higher than that

from the MTP assay. The exception to this was in the case of MBEC of CAZ for *K. pneumoniae* which was lower in the MBEC<sup>TM</sup> assay.

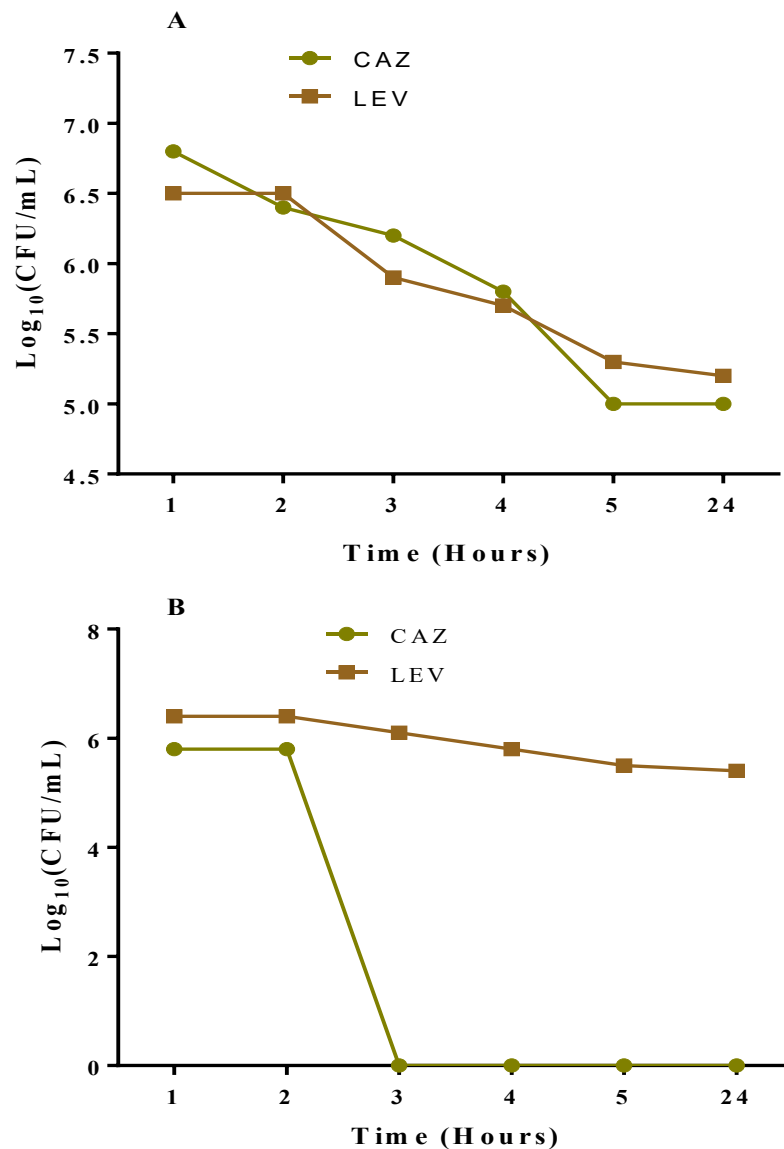
#### **4.8 Isolation of persister cells in Gram–negative DFU isolates after antibiotic challenge**

The ability of test strains, *K. pneumoniae* and *P. mirabilis* to produce persister cells was assessed using 3 methods described by Keren *et al.* (2004). Two phases of the bacterial growth cycle, logarithmic and stationary, were challenged with ceftazidime and levofloxacin respectively (Spoering and Lewis, 2001). In a 4th method persister cells were isolated from *K. pneumoniae* and *P. mirabilis* biofilm in a two–fold dilution assay with levofloxacin of working concentration of 5120 µg/mL. LEV also targets non–growing cells and biofilm cultures (Brooun *et al.*, 2000). To determine whether persister cells are genetic variants of ‘wild–type’ or ‘parent cells’, two tests were performed. The susceptibility of persister cells and their planktonic (parent) cells were tested against ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin. The second test was the use of PCR amplification to determine differences in gyrase B gene of biofilm–extracted persister cells and parent cells.

##### **4.8.1 Time–dependent isolation of persister cells from DFU isolates**

*K. pneumoniae* and *P. mirabilis*, clinical (test) strains used in this study have been found to be strong biofilm producers (Table 4.3) and are partially tolerant to CAZ and LEV. The antibiotic concentration used for the time–dependent assay was 25 times the MIC for *K. pneumoniae* and *P. mirabilis* (EUCAST, 2014). At designated time points (1, 2, 3, 4, 5 and 24 hours) cell numbers were estimated as CFUs and graphs of Log<sub>10</sub> CFU/mL against time were plotted. The time – kill graph in Figure 4.6A shows an initial killing of exponential phase *K. pneumoniae* cells when challenged with CAZ and LEV. A fraction of the population of *K. pneumoniae* cells was not killed after 4 hours of incubation. However, a killing plateau, which was indicated by either slow killing of cells or the presence of subpopulation dormant/antibiotic tolerant cells called persister cells, was observed from early stationary phase to the decline phase. The killing pattern was however similar to that previously observed for other bacteria that showed typical killing patterns characteristic of the presence of persister cell subpopulation, a fraction of the population that tolerated killing by

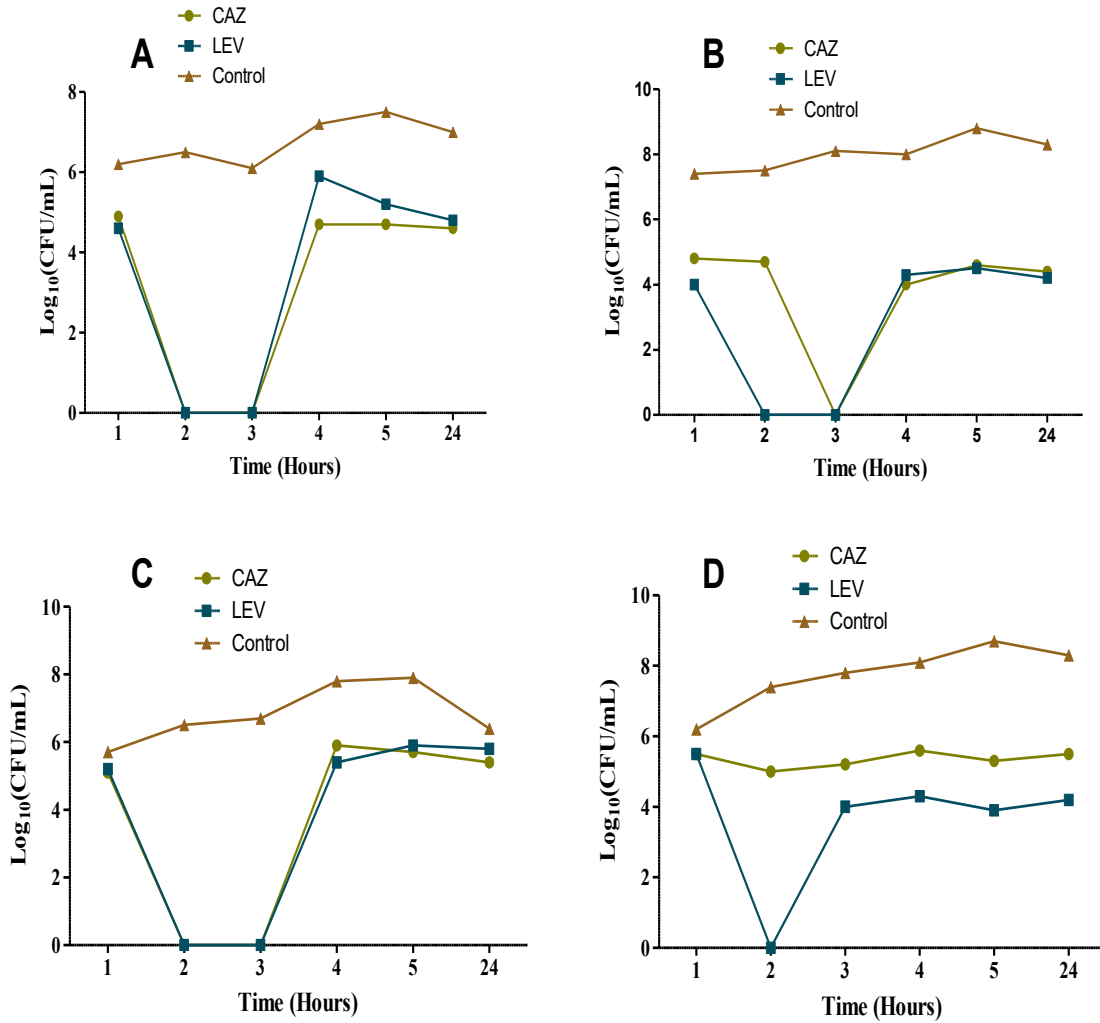
ampicillin, gentamicin and ofloxacin (Keren *et al.*, 2004; Keren *et al.*, 2012; Kint *et al.*, 2012; Lewis, 2007; Spoering and Lewis, 2001). Unlike LEV, *P. mirabilis* persister cells produced after challenge with CAZ were undetectable when spot-plated on agar after 3 hours of incubation (Figure 4.6B). However, when exponential phase *P. mirabilis* was challenged with LEV, a gradual killing pattern was observed that stabilised from early stationary phase to the decline phase.



**Figure 4.6** Time-dependent isolation of persister cells using ceftazidime and levofloxacin (100  $\mu\text{g}/\text{mL}$ ). (A) *K. pneumoniae* and (B) *P. mirabilis* planktonic cells were challenged with ceftazidime and levofloxacin and the amount of persister cells produced were monitored every hour for 4 hours.

#### 4.8.2 Growth–stage dependent production of persister cells from DFU isolates

It has been suggested that, the persister cell subpopulation of a bacterial culture are generated during growth phase before any antibiotic treatment (Balaban *et al.*, 2004; Balaban *et al.*, 2013; Keren *et al.*, 2004). However, the number of persister cells produced during the stationary phase is dependent on growth conditions such as age of inoculum/culture, types of bacterial strain (either slow–growing or rapid–growing), type of culture media and the types of antibiotics used for the challenge assays (Luidalepp *et al.*, 2011). For example, Luidalepp *et al.* (2011) observed that, the levels of persister cells produced by both 18–hour stationary phase *E. coli hipA7* and its wild–type challenged with ampicillin and incubated for extended period of time decreased. On the other hand, all cells were killed when challenged with amikacin during growth resumption experiments with fresh LB broth (Luidalepp *et al.*, 2011). In the current study, the difference between persister cell production during exponential and stationary phases of growth were challenged with CAZ and LEV and assessed. In the growth–stage dependent assays for persister cell isolation from *K. pneumoniae* and *P. mirabilis*, the concentration of CAZ and LEV was doubled (200 µg/mL) due to high levels of non–dividing cells produced during the stationary phase of bacterial growth. The method was slightly modified to incorporate 2 time points; antibiotic challenge at (a). 1 hour after incubation and (b). 3 hours after incubation to determine if extended incubation periods affect the levels of persister cell production. The number of persister cells produced was then estimated as CFU/mL and plotted against time.



**Figure 4.7** Growth–state dependent isolation of persister cells. *K. pneumoniae* and *P. mirabilis* were challenged with 200 µg/mL of CAZ and LEV at (A) and (C) 1 hour and; (B) and (D) 3 hours post–incubation with CAZ and LEV and samples removed at designated time points (hourly) and CFU determined. Control was made up of unchallenged *K. pneumoniae* or *P. mirabilis* sampled at the same time as the test strains.

Aliquots of growing *K. pneumoniae* cells were removed every hour and challenged with CAZ and LEV. It was observed that *K. pneumoniae* cells were quickly killed by CAZ and LEV and persister cells were undetected for 2 hours (Figure 4.7A). After the two–hour dormancy, *K. pneumoniae* cells were detected again and stabilised afterwards. When samples were removed, and challenged after every 3 hours, persister cells produced by LEV–challenged *K. pneumoniae* cells were undetectable at 2 time points whereas persister cells produced by CAZ–challenged *K. pneumoniae* cells were undetectable at only one–time point (Figure



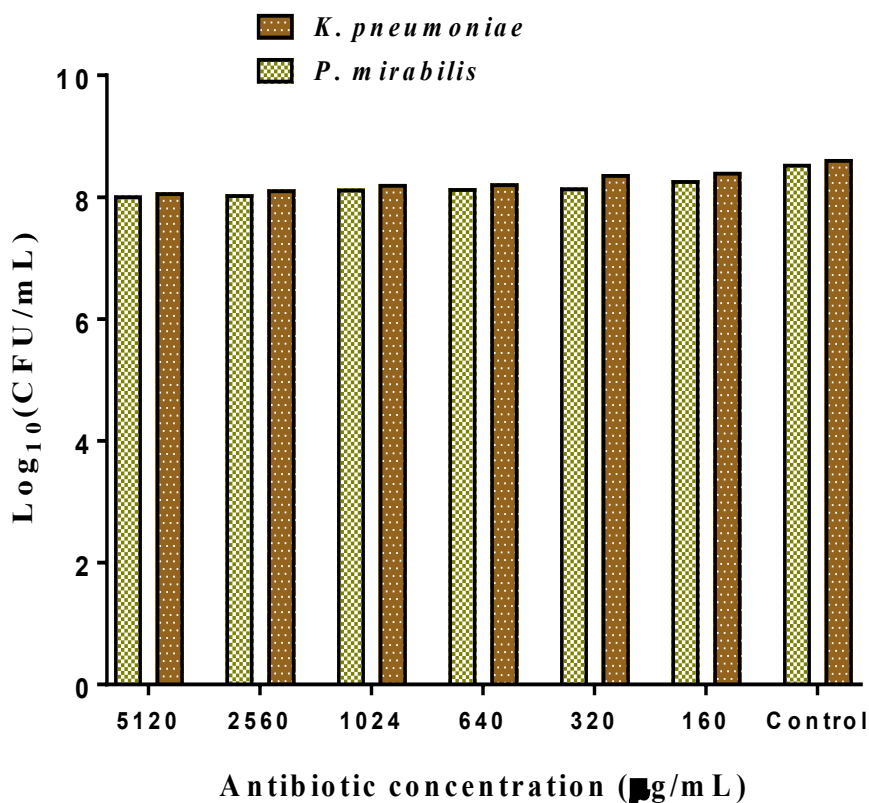
4.7B). Similarly, in Figure 4.7C), persister cells produced by CAZ and LEV–challenged *P. mirabilis* cells were undetectable at 2 time points. On the other hand, persister cells produced by LEV–challenged *P. mirabilis* following 3 hours of initial incubation were undetectable at only one–time point after which the levels reached a plateau. The patterns of growth of *K. pneumoniae* and *P. mirabilis* persister cells illustrated in Figure 4.7A, B, C and D are slightly different from that observed with *E. coli* by Keren *et al.* (2004). This can be explained as probably increase in the number of uncultivable cells in broth due to the length of the stationary phase which resulted in late onset of growth after being plated–out on agar. In future, growth – dependent persister cell isolation assays could incorporate biosensor reporters such as the green fluorescent protein or its derivatives which could bind to non–dividing or uncultivable cells and determined by fluorescent microscopy (Luidalepp *et al.*, 2011; Yuste, 2005).

#### **4.8.3 Isolation of persister cells from biofilms produced by DFU isolates**

In this assay, the production of persister cells from biofilm cultures was determined by estimating the survival (as CFU/mL) of *K. pneumoniae* and *P. mirabilis* cells after challenge of biofilm cultures with antibiotic. In order to confirm that persister cells were produced after antibiotic challenge of *K. pneumoniae* and *P. mirabilis* biofilms with LEV, persister cells were harvested from biofilm cultures using the method outlined in section 2.5.4. *K. pneumoniae* and *P. mirabilis* wild–type or planktonic parent cells and their biofilm – derived persister cells counterparts were tested for their antibiotic susceptibility against a selection of antibiotics in order to confirm the hypothesis that, the biofilm–derived persister cells are not genetic variants of the wild – type; results of which are shown in section 4.7.4.1.

In Figure 4.8, it was observed that, LEV at high concentration (5120 µg/mL, which is 1280 times the MIC) was unable to completely eradicate *K. pneumoniae* and *P. mirabilis* biofilms and/or persister cells after 3 hours of incubation. At 5120 µg/mL, LEV eradicated 71 and 70% of *K. pneumoniae* and *P. mirabilis* biofilms respectively which was found to be statistically significant by two–way ANOVA ( $p = 0.0031$ ). It can be observed in Figure 4.8 that biofilm cultures of *K. pneumoniae* and *P. mirabilis* showed significant tolerance to killing by LEV with Log<sub>10</sub> CFU/mL survival of 8.63 and 8.55 compared to planktonic cells in

the exponential (5.2 and 5.4) and stationary phases (4.8 and 5.8) of *K. pneumoniae* and *P. mirabilis* growth respectively in Figures 4.6 and 4.7.



**Figure 4.8** Isolation of persister cells from biofilms challenged with different concentrations of levofloxacin (160–5120 µg/mL). Total viable cell count recovered from levofloxacin agar plates ranged from  $1.2 \times 10^8$  to  $2.6 \times 10^8$  CFU/mL.

#### 4.8.4 Persister cells are not genetic mutants of wild-type cells

To confirm that persister cells are not genetic mutants of the wild-type, a series of assays were performed. Biofilm-derived persister cells were tested for their antibiotic susceptibility patterns against 5 antibiotics and compared with the wild-type. Persister cells produced after *K. pneumoniae* and *P. mirabilis* planktonic cells were challenged with LEV were tested in a time-dependent assay to determine whether the mechanisms that lead to persister cell formation were inherited from parent cells. In another assay, biofilm-derived persister cells were grown in a conventional 96-well MTP, stained with 0.1% crystal violet and the OD<sub>570</sub> values compared to that obtained in Table 4.3 (results not included). Lastly, PCR

amplifications were performed to compare the gyrase B (*gyrB*) genes in biofilm-derived *K. pneumoniae* and *P. mirabilis* persister cells with the wild-type.

#### 4.8.4.1 Antibiotic susceptibility testing of planktonic and biofilm-derived persister cells

The antibiotic susceptibility testing of *K. pneumoniae* and *P. mirabilis* planktonic and biofilm-derived persister cells were performed using 5 selected antibiotics (ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin). The susceptibility patterns of *K. pneumoniae* and *P. mirabilis* planktonic and biofilm-derived persister cells were compared using the BSAC and EUCAST breakpoints as reference. EUCAST breakpoints were used for some specific strains in the *Enterobacteriaceae* family where BSAC had no established breakpoints for the interpretation of their zones of inhibitions for some antibiotics. The susceptibility profiles in Table 4.13 show that biofilm-derived *K. pneumoniae* and *P. mirabilis* persister cells retained the same susceptibility profiles as the wild-type following antibiotic challenge and regrowth in fresh medium.

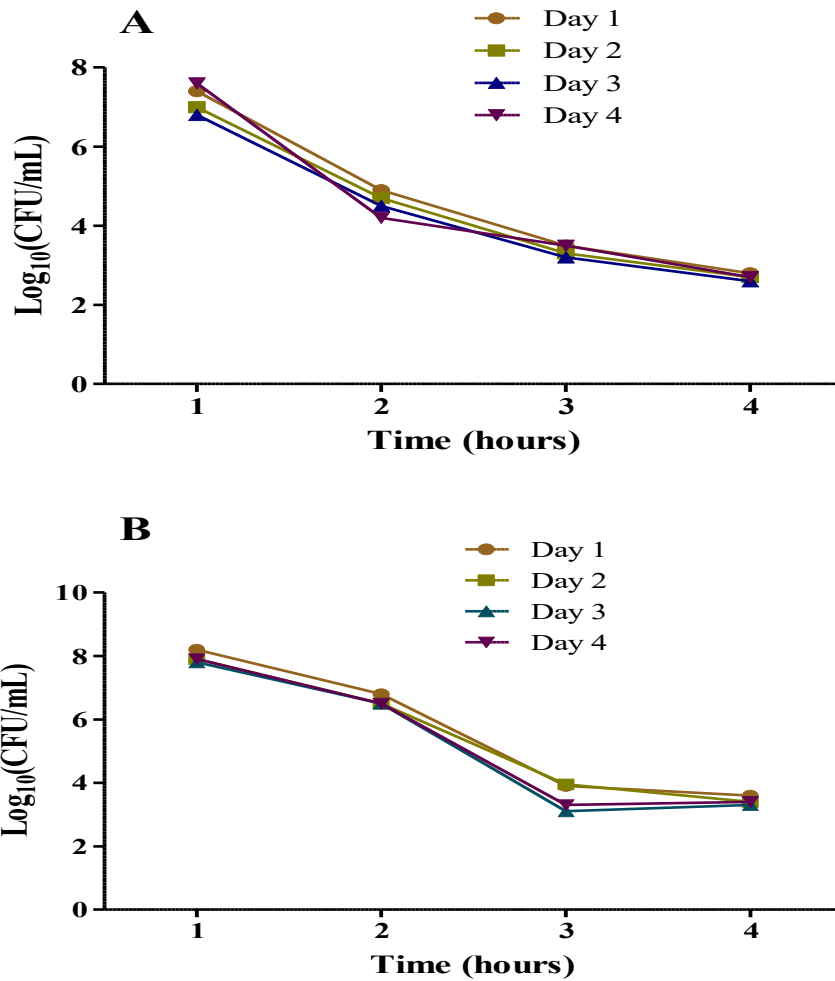
**Table 4–13** Antibiotic susceptibility profile of *K. pneumoniae* and *P. mirabilis* and their corresponding persister cells

Antibiotic	<i>K. pneumoniae</i> Diameter of zone of inhibition (mm)		<i>P. mirabilis</i> Diameter of zone of inhibition (mm)		Zone diameter breakpoint (BSAC) (mm) R ≤ I ≤ S
	Planktonic	Persister	Planktonic	Persister	<i>Enterobacteriaceae</i>
Ampicillin, 10 µg	10	10	10	11	14 ≤ – ≥ 15
Ceftazidime, 30 µg	5	5	12	15	19 < 20–21 ≥ 22 <sup>a</sup>
Ciprofloxacin, 5 µg	22	22	3	3	19 < 20–21 ≥ 22 <sup>b</sup>
Gentamicin, 10 µg	11	11	12	13	16 ≤ 17–19 ≥ 20
Levofloxacin, 5 µg	22	22	22	21	19 < 20–21 ≥ 22 <sup>c</sup>

a, b, c – EUCAST zone diameter breakpoints for ceftazidime, ciprofloxacin and levofloxacin (EUCAST, 2014).

#### **4.8.4.2 Persister heritability assay**

This assay was performed to determine whether persister cells are genetically different from the wild-type and their increased survival was transferrable to their progeny (Wolfson *et al.*, 1989). The time-dependent assay (Figure 4.9) following 4 hours of incubation after antibiotic challenge showed a similar killing pattern observed for the wild-type demonstrated in Figure 4.6 above. The similar pattern of killing in both independent assays shown in this study is suggestive that mechanisms underlying persister cell formation are not heritable. A similar observation was made by Keren *et al.* (2004) using ampicillin for the time-dependent killing assay.

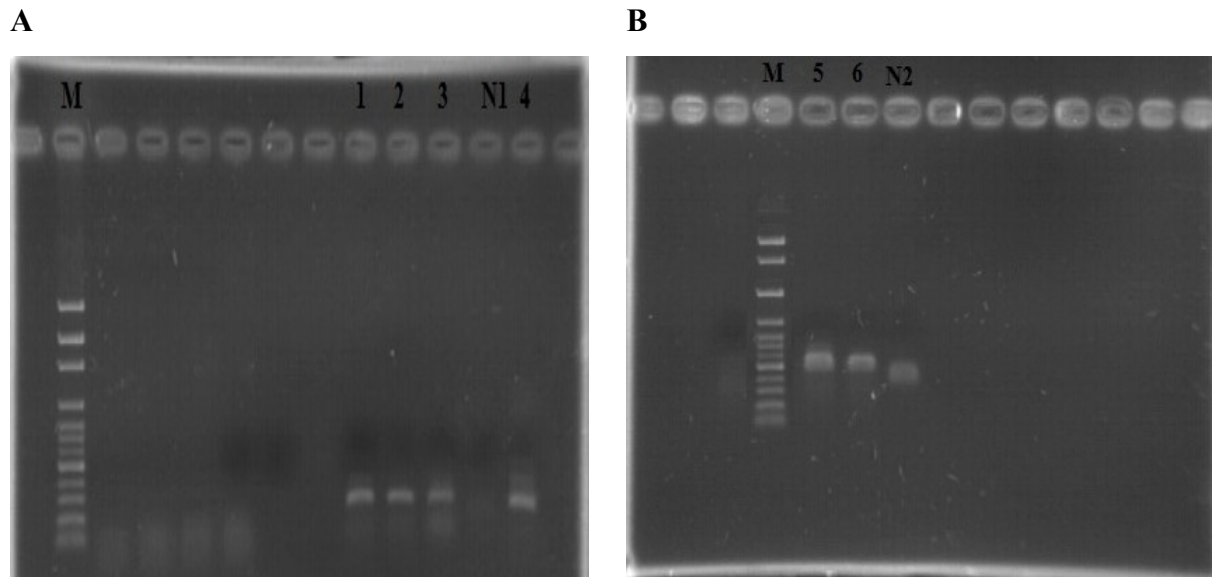


**Figure 4.9** Persister heritability assay showing exponentially grown (A) *K. pneumoniae* and (B) *P. mirabilis* cells challenged with LEV. Time-dependent killing of the bacterial cells over the 4 day-period was found to be statistically significant ( $p < 0.0001$  by two-way ANOVA).

#### 4.8.4.3 Genotypic characterisation of biofilm-derived persister cells and wild-type strains

The amplification of the *gyrB* gene in both *K. pneumoniae* and *P. mirabilis* planktonic and biofilm-derived persister cells showed no differences in the amplified gene products. Primers designed to target the *gyrB* gene in both the persister cell DNA and wild-type DNA amplified partial sequences of the gene of the same size as resolved by gel electrophoresis (Figure 4.10). The results were also confirmed by PCR purification and DNA sequencing. The sequenced partial gene products were aligned with speciated strains deposited in the

databases to look for similarity between the sequenced. There was 100% alignment between the genes which also suggests that, there was no genotypic difference between the *gyrB* genes of *K. pneumoniae* and *P. mirabilis* biofilm – derived persister cells and their wild –type counterparts (Appendix C2).

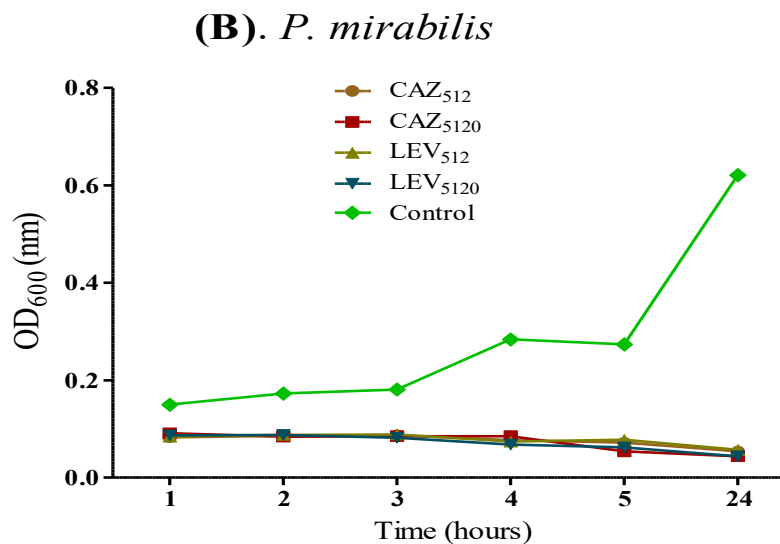
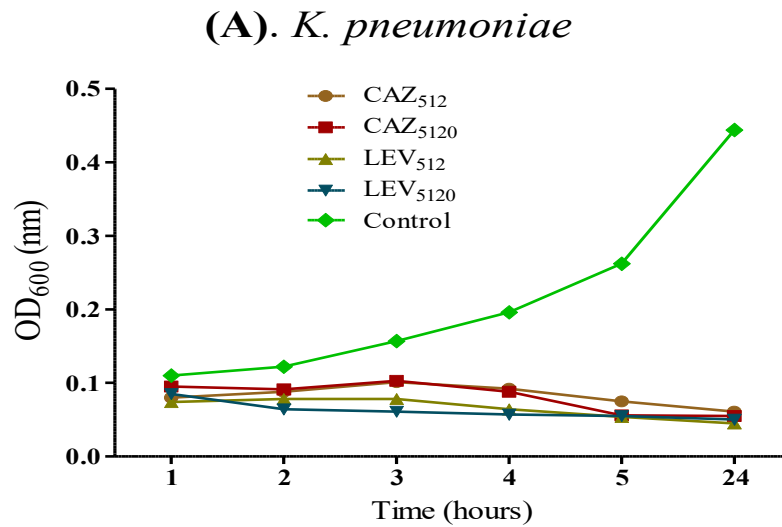


**Figure 4.10** PCR amplification of *gyrB* partial gene of both wild–type and persister cells of; (A) *P. mirabilis* (200 bp) and (B) *K. pneumoniae* (509 bp) and. Lanes: M – DNA marker (100 bp); 1 and 2 represent of wild–type *K. pneumoniae* strains; 3 and 4 represent *K. pneumoniae* persister; 5 represents wild–type *P. mirabilis* and 6 represents *P. mirabilis* persister; N1 and N2 are negative controls.

#### 4.9 *Quasi-Vivo*® QV500 chamber assay – time–dependent biofilm eradication

This is the first time the *Quasi-Vivo*® system, a continuously flow system, has been used for a bacterial assay. In this study, the *Quasi-Vivo*® system was used to assess the effect of antibiotic treatment of biofilm on coverslips in a continuous flow system in a time–dependent killing assay. In this assay, biofilm eradication was monitored as a function of the measure of the direct effect of antibiotic challenge on dispersed bacteria in suspension on biofilms seeded on coverslips by spectrophotometry and viable cell count (CFU/mL). Residual biofilm on the coverslips in the QV500 chambers were qualitatively determined after antimicrobial challenge by epifluorescence microscopy after staining with LIVE/DEAD® BacLight

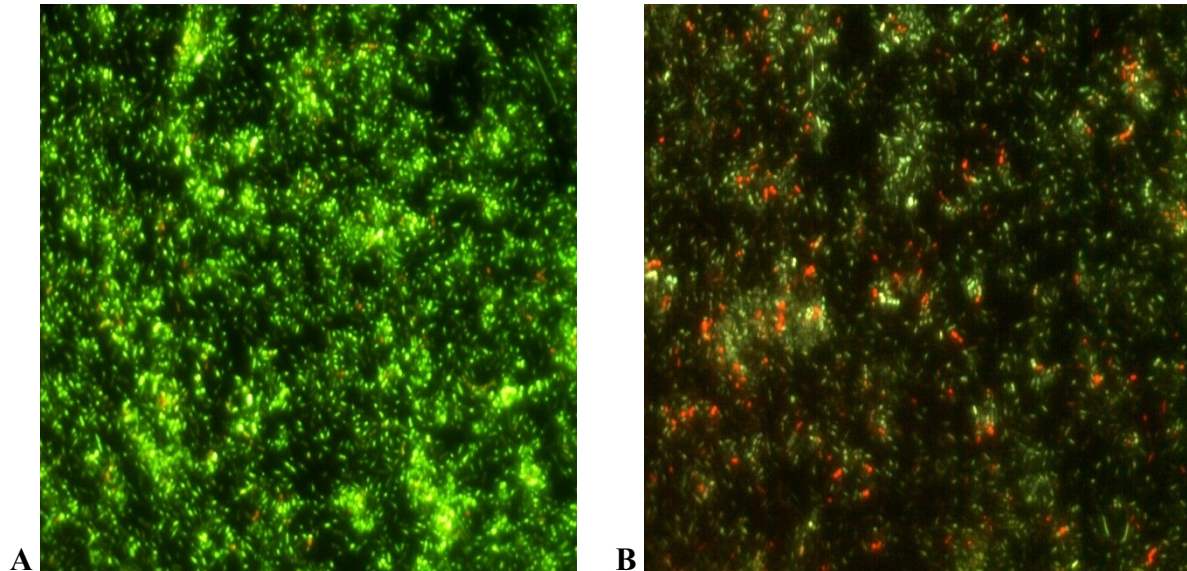
Bacterial Viability kit (Molecular Probes, USA). It was observed that, the amount of *K. pneumoniae* and *P. mirabilis* cells dispersed from the biofilm decreased steadily over time after the antibiotic challenge (Figures 4.11A and B). A direct relationship was observed between OD<sub>600</sub> and CFU/mL across the selected time points.



**Figure 4.11** Time-dependent eradication of; (A) *K. pneumoniae* and (B) *P. mirabilis* biofilms using the QV500 chamber assay. Amount of dispersed bacterial cells in solution ( $OD_{600}$ ) decreased steadily over the time-period (two-way ANOVA,  $p = 0.0017$  and  $p = 0.0011$  respectively). Control was made up of a cover slip of *K. pneumoniae* or *P. mirabilis* biofilm passed through the chamber and sampled at the selected time points without antibiotic challenge.



Epifluorescence microscopy of the LIVE/DEAD<sup>®</sup> stained biofilm coverslips showed the extent of biofilm eradication which was indicated by dead bacterial cells (shown as red cells) and the voids between the microcolonies (Figure 4.12B).



**Figure 4.12** Epifluorescence images of BacLight LIVE/DEAD<sup>®</sup> stained coverslips of biofilms images of; (A). 24–hours old *P. mirabilis* biofilm without antibiotic challenge and; (B) *P. mirabilis* biofilm challenged with 5120  $\mu\text{g}/\text{mL}$  levofloxacin and showing dead cells (in red).

## 4.10 Discussion

*K. pneumoniae* and *P. mirabilis* strains have been well studied as members of the *Enterobacteriaceae* family with medical importance. *K. pneumoniae* and *P. mirabilis* are environmental organisms and part of the normal flora of the skin and intestines which have been associated with both community- and hospital-acquired conditions such as pneumonia, urinary tract infections (UTIs) and septicaemia (Chong *et al.*, 2013; Podschun and Ullmann, 1998). Their presence in opportunistic infections such as wounds and burns has also been well studied (Gardner *et al.*, 2013; Gjødsbøl *et al.*, 2006; James *et al.*, 2008; Malik *et al.*, 2013; Oates *et al.*, 2012; Rhoads *et al.*, 2012). This work presented here established that, the growth cycles of *K. pneumoniae* and *P. mirabilis* (designated as strains 028b and 005 respectively) were similar to that of the reference *E. coli* NCTC 10418 strain. The estimations of bacterial biomass at each stage of the growth cycle of the 3 strains were comparable (Figures 4.1 and 4.2). The growth cycles of *K. pneumoniae* and *P. mirabilis* suggested that they could be used as model strains in the studies of bacterial physiology and metabolism and their response to external stresses.

In the coaggregation assay, *K. pneumoniae* and *P. mirabilis* showed their ability to autoaggregate and coaggregate with other DFU isolates (Table 4.1 and 4.2). Their ability to autoaggregate and/or coaggregate after 2 hours of incubation with little or no changes in coaggregation score up to 24 hours of incubation suggests that they have the ability to form and sustain biofilm formation (Hill *et al.*, 2010). The formation of coaggregation phenotypes by wound isolates is an important step in biofilm formation *in vivo* (Rickard *et al.*, 2003; Rickard *et al.*, 2004). Coaggregation reaction was stronger among some coaggregation pairs than others with both higher visual and percentage (quantitative) scores. These higher scores are consistent with specificity between cell surface appendages that mediate bacterial adhesion to one another as evident in dental plaques (Kolenbrander, 2000; Stinson *et al.*, 1991). Low coaggregation scores among some wound isolates do not rule out bacterial interactions within these wounds (Hill *et al.*, 2010). This is because, bacteria colonising wounds are not exposed to high shear forces as evident in the oral cavity where bacteria exhibit stronger coaggregation reactions with higher scores (Hill *et al.*, 2010; Shen *et al.*, 2005).

In this study, *K. pneumoniae* and *P. mirabilis* were classified as strong biofilm producers using the method described by Merritt *et al.* (2011) and Pye *et al.* (2013). Biofilm formation by *K. pneumoniae* and *P. mirabilis* was also confirmed by the production of the extracellular polymeric substance (EPS) which was demonstrated by 2 staining techniques and visualised by epifluorescence microscopy (Figure 4.3). However, biofilm production by *K. pneumoniae* and *P. mirabilis* and other wound isolates was found to be affected by changes external to the bacterial microenvironment such as the incubation temperature, pH and nutrient concentration of the growth media.

It was observed that biofilm production among some isolates notably, 021a *P. mirabilis*, 038 *P. aeruginosa* and test strains 028b *K. pneumoniae*, and 005 *P. mirabilis*, was inconsistent with changes in pH as they did not relate to growth optima. However, the clinical test isolates used in this study, 028b *K. pneumoniae* and 005 *P. mirabilis* showed significant growth at pH 4 and 10 respectively (Figure 4.4A). *K. pneumoniae* has been previously found to grow optimally at pH 5.5 – 7.0 (Jones *et al.*, 2015). A similar observation was made in the temperature assay (Figure 4.4B and C). *P. aeruginosa* for example has previously been found to produce more biofilm at high pH which was also observed in this study (Harjai *et al.*, 2005). Two major regulatory genes, *rpoS* and *algT* responsible for initiating stress response during biofilm formation have been found in *P. aeruginosa* and *E. coli* (Cochran *et al.*, 2000). These genes mediate physiological changes that protect the biofilm structure from environmental stresses such as cold shock, heat shock, oxidative stress as well as other chemical agents. The ability of *P. aeruginosa* to form biofilm under such extreme environmental conditions is due to the production of the extracellular polysaccharide called alginate (Cotton *et al.*, 2009; Moller *et al.*, 2008; Stapper *et al.*, 2004). Alginate in itself is not required for biofilm formation but has a role in providing structural protection for the biofilm to resist environmental stress as well as antibiotic killing (Cotton *et al.*, 2009; Stapper *et al.*, 2004). *AlgT* and *RpoS* are also known to coordinate responses that make biofilms less susceptible to treatment with oxidative agents such as hydrogen peroxide (Cochran *et al.*, 2000). The structural integrity of *E. coli* biofilm is also maintained by the *RpoS* sigma factor through the regulation of genes such as *nlpI* and *osmB* that are involved in membrane resealing after exposure to external stresses (Charoenwong *et al.*, 2011; Cochran *et al.*, 2000).

Jones *et al.* (2015) mentioned in their recent review that the effect of pH on wound isolate on growth and biofilm formation has not been specifically considered by any study. This current study however, is one of the few that has considered the *in vitro* effects of pH and environmental conditions on wound isolates other than the usual pathogens such as *E. coli*, *P. aeruginosa* and *S. aureus*. Against the backdrop that *P. aeruginosa* and *E. coli* possess regulatory genes which can be upregulated to protect them against changes in environmental conditions, it can be suggested that, *K. pneumoniae* and *P. mirabilis*, may possess similar regulatory factors hence their ability to produce significant biofilm at pH of 4 and 10 respectively. However, little is known about any intrinsic regulatory systems that enable *K. pneumoniae* and *P. mirabilis* to withstand such degrees of environmental stress. In this study, the determination of such regulatory factors in *K. pneumoniae* and *P. mirabilis* was not further investigated as it was beyond the budget of this project. It can also be suggested that their ability to form strong coaggregation phenotypes (Tables 4.1 and 4.2) and synthesise polysaccharides and EPS (Figure 4.3) during biofilm formation may play a part in their ability to withstand some external stresses. There was a positive correlation between the production of biofilm and nutrient concentration. This is very significant as bacterial growth media especially LB contain both tryptone and yeast extract and other nutrients such as amino acids, vitamins, nitrogen and carbon sources with carbon–nitrogen ratio of 0.26:1 which are essential requirements for bacterial growth (O’Kennedy *et al.*, 2000; Ramli *et al.*, 2012).

Although biofilm production was markedly reduced in some isolates during the pH, temperature and nutrient concentration assays, they were not completely inhibited. However, the regulation of biofilm formation and development in chronic wounds is a complex process that is species–specific and influenced by both the genetic make–up of the bacterial species involved, the environmental conditions and host immune responses prevailing at the site of infection (Lopez *et al.*, 2010; Ramli *et al.*, 2012). It has been noted that, the slow rate of healing of chronic wounds is due to the alkaline nature of the wounds (Schneider *et al.*, 2007). However, as a wound progress towards healing, the pH also shifts towards acidity (Shukla *et al.*, 2007). This suggests that, pH plays a role in wound healing and can serve as a tool for monitoring non–healing wounds. In the future, treatment strategies targeting non–healing wounds could consider the use of biocides or antimicrobials that can reduce the pH of the wound environment in order to facilitate healing.

The antibiotic susceptibility patterns (using the disc diffusion method) of planktonic cells of all DFU isolates including test strains *K. pneumoniae* and *P. mirabilis* have previously been determined (section 3.3 in Chapter 3). In this chapter, the ability of 5 antibiotics including ceftazidime and levofloxacin, to inhibit and/or eradicate biofilms was assessed using two microtitre plate based assays. Ceftazidime and levofloxacin were selected for subsequent assays and for the analyses of MIC, MBC and MBEC results obtained from both conventional MTP and MBEC™ assays. Their modes of action also make them suitable choices for investigation bacterial biofilm and isolation of persisters (Brooun *et al.*, 2000; Keren *et al.*, 2004, Keren *et al.*, 2012 Spoering and Lewis, 2001). Ceftazidime (CAZ), a third-generation cephalosporin with a beta-lactam mode of action is less susceptible to the action of beta-lactamases (Lagacé-Wiens *et al.*, 2014). Levofloxacin, like all other quinolones has good tissue penetration and is effective against slow-growing bacterial cells. The high antibiotic efficacy of levofloxacin against slow-growing cells has been attributed to its good pharmacokinetics, i.e., rapid and complete absorption and high bactericidal activity. It is also thought that, like ciprofloxacin, efflux pumps do not export levofloxacin hence its increased bactericidal activity (Brooun *et al.*, 2000). In the bacterial cell, Levofloxacin inhibits the 2 type II topoisomerases; DNA gyrase and topoisomerase IV, leading to DNA separation after replication (Drlica and Zhao, 1997).

In the MTP assay, levofloxacin at  $\geq 160 \mu\text{g/mL}$  (40 times the MIC) was the most effective antibiotic to inhibit 63% of *K. pneumoniae* biofilm (Table 4.4). However,  $\geq 640$  times the MIC ( $\geq 2560 \mu\text{g/mL}$ ) was needed by ceftazidime to inhibit 62% of *K. pneumoniae* biofilm. The concentration of CAZ and LEV ( $\geq 640 \mu\text{g/mL}$ ) that inhibited *P. mirabilis* biofilm by 65% each was 160 times the MIC. The MBEC of CAZ and LEV needed for complete eradication of *K. pneumoniae* and *P. mirabilis* biofilms was  $\geq 5120$  and  $2560 \mu\text{g/mL}$  ( $> 640$  times their MICs) (Table 4.5). It can be suggested that both CAZ and LEV have increased efficacy against both *K. pneumoniae* and *P. mirabilis* cells during the lag and logarithmic phases of their growth cycles (Desai *et al.*, 1998). However, their respective efficacies reduce during the stationary growth phase where there is decreased metabolic rate, slow growth and formation of biofilm hence the increase in their MICs. Also, the synthesis of the EPS during biofilm formation serves as a barrier that prevents the entry of antibiotics into the embedded biofilm (Lopez *et al.*, 2010; Mah and O'Toole, 2001). The increased MIC of CAZ and LEV in inhibiting *K. pneumoniae* and *P. mirabilis* biofilms may also be due to the presence of

multiple antibiotic resistance genes (Mah and O'Toole, 2001; Singla *et al.*, 2013). In the work presented here, planktonic cells of both *K. pneumoniae* and *P. mirabilis* were susceptible to LEV but resistant to CAZ.

Mixed biofilms have been found to be more prevalent in the oral cavity where their interactions benefit one or all partners (Kolenbrander *et al.*, 2010; Stacy *et al.*, 2014). In this study, however, the coaggregation phenotypes and corresponding scores suggest that DFU isolates are capable of forming mixed biofilms through bacterial cell-to-cell interactions. It has been established that bacteria involved in biofilm formation engage in active communication, coordination and collective behaviour in mature biofilm communities (Dow *et al.*, 2003; Dunne, 2002; Hammer and Bassler, 2003; Stoodley *et al.*, 2002). However, these biofilm communities are more complex and diverse than they were thought to be. Complex adherence kinetics and dynamics in the physiological environment of polymicrobial biofilms have been found to affect the development of microcolonies and spatial biofilm structures (Ibusquiza *et al.*, 2012; Xavier and Bassler, 2005a; Xavier and Bassler, 2005b). Fight-and-flight responses and competitive effects have also been found to affect specific and non-specific adherence between 2 strains during mixed biofilm formation leading to differential responses to environmental stresses like antibiotic treatment and antimicrobial production (Burmølle *et al.*, 2006; Mellefont *et al.*, 2008; Stacy *et al.*, 2014). For instance, Stacy *et al.* (2014) demonstrated an *in vitro* bacterial fight-and-flight response in dental plaque and suggested that such fight-and-flight responses enhance the virulence of pathogenic bacteria in a polymicrobial infections. In their study, Stacy *et al.* (2014) observed that, *Streptococci gordonii* promoted the growth and virulence of the oral pathogen *Aggregatibacter actinomycetemcomitans* by producing L-lactate as a carbon source and in effect produced hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). They observed that *A. actinomycetemcomitans* responded to the high levels of the antimicrobial H<sub>2</sub>O<sub>2</sub> either by a catalase – mediated enzymatic detoxification (i.e., fight response) of H<sub>2</sub>O<sub>2</sub> or by spatial dispersion (i.e., flight response) of the *A. actinomycetemcomitans* biofilm through the action of an enzyme called dispersin B. Mellefont *et al.* (2008) also noted that, competitive effects otherwise known as the 'Jameson Effect' among bacterial pairs in a mixed culture, may lead to the production of growth inhibitors by one strain against the other. In addition to the Jameson Effect, other more complex interactions in a mixed culture lead to competition for nutrients, pH reduction or increase and differential use of available substrate. The above variations and some other

factors may account for the complex responses of mixed biofilms to antibiotic/antimicrobial treatment compared to monospecies biofilms.

Biofilm estimation results following MIC and MBEC determinations (as shown in Table 4.12) either by the conventional MTP or MBEC™ assay may be due to the sensitivity of each assay. Due to its many modifications, the conventional MTP assay may be less sensitive with a wider margin of error compared to the calibrated MBEC™ assay. Hence the results obtained using the *in vitro* MBEC™ assay may provide more accurate analysis comparable to *in vivo* situations.

The differences between the methodologies used in the conventional MTP and MBEC™ assays pose a challenge in the quantitative comparisons of the independent results obtained by the 2 assays. Some of the differences include the volume and the inoculum size of the starting inoculum suspension for both assays. In the MTP assay, the volume of starting inoculum suspension range from 100 to 200  $\mu\text{L}$  while that of the MBEC is fixed at 150  $\mu\text{L}$ . In the MTP assay, biofilm estimation is performed on biofilms formed in the bottom and walls of the wells whereas in the MBEC™ assay biofilms formed on pegs suspended from the lid of the MBEC™ plate are estimated and that formed in the bottom of the wells are discarded. The starting inoculum suspension for the MBEC™ assay is always  $3.0 \times 10^8$  CFU/mL while that of the MTP assay is usually 0.5 McFarland (i.e., approximately  $1.5 \times 10^8$  CFU/mL) with occasional references to the optical densities (Ceri *et al.*, 1999; Harrison *et al.*, 2005; Merritt *et al.*, 2011; O'Toole *et al.*, 1999; O'Toole, 2011; Stepanovic *et al.*, 2001). Against the backdrop of differences in initial inoculum suspension volumes and cell concentrations, the MTP assay may result in the formation of more biofilms than in the MBEC™ assay hence requires antibiotics with higher concentrations to inhibit and/or eradicate biofilm formation (Table 4.12). Therefore, the use of different starting inoculum suspension volumes and inoculum sizes prevents comparison of biofilm estimations between the MTP and MBEC™ assay. The incubation step of the MBEC™ involves either rotation at 5 rocks per minute (for the trough–base assay) on a rotor or 110 rpm (for the 96–well base assay) in a shaking incubator. The incubation step of the conventional MTP assay is undefined and subject to modifications based on the growth requirements of the bacteria under investigation; whether slow– or fast– growing. Also, the washing step in the MTP assay involves a rigorous and careful washing of the 96–well plate submerged in a bowl of

water (Merritt *et al.*, 2011). Discrepancies in the final absorbance readings of the crystal violet stained biofilm can be attributed to other factors such as the washing steps as some biofilms are likely to be washed off during the washing of unbound cells after overnight incubation of the MTP plate (Merritt *et al.*, 2011; Pye *et al.*, 2013). This implies that bacterial isolates classified either as weak, moderate or strong biofilm producers using MTP assay may be classified otherwise using the MBEC™ assay. The above-mentioned variations of the MTP assay suggest that the MTP assay may be an unreliable assay for biofilm estimations and subsequent classification of bacterial isolates as biofilm producers.

In this study, some of the modifications made to the MTP assay outlined in section 2.4.1 included, fixation of the biofilm after the appropriate incubation period with 4% formaldehyde before the initial washing step. Also, in place of the rigorous washing step in a bowl, a careful aspiration of the unbound cells in suspension by means of a pipette can be done. The last modification of this assay is the choice of a solubilising agent (30 – 33% acetic acid, 95% ethanol, 80% ethanol/20% acetone, 100% dimethyl sulfoxide) which depends on the type of biofilm producing strain (Merritt *et al.*, 2011). The MBEC™ assay is calibrated by the manufacturer with no external modifications allowed during the assay. The classification of bacterial strains as biofilm producers is based on the determination of their CFU/mL. Biofilm estimation in the MTP assay involves the absorbance reading at OD<sub>570</sub> (in nanometres) of crystal violet-stained biofilm in the bottom and on the walls of the 96-well plate. In the MBEC™ assay, biofilm growth estimation on each peg was removed by sonication of the formed biofilm into a recovery medium followed by spot-plating of diluted cells on agar to determine their CFU/mL. For the purpose of this study, the definitions for MIC and MBEC are given as complete inhibition or eradication of biofilm at OD<sub>570</sub> < 0.05 and OD<sub>650</sub> < 0.1 for the MTP and MBEC™ assays respectively. Where there were no complete inhibition or eradication of biofilm, percentage inhibition or eradication were provided to define the extent of biofilm inhibition or eradication at the concentrations of the antibiotics/antimicrobials used.

In this study, the disc diffusion assay results of antibiotic susceptibility testing (Figure 3.5, section 3.3.1) suggest that, both ceftazidime and levofloxacin were more effective against *K. pneumoniae* and *P. mirabilis* planktonic cells than the other antibiotics used. However, neither ceftazidime nor levofloxacin could completely inhibit or eradicate biofilms. Biofilm



eradication has been a major dilemma (Lewis, 2007). Though the development of resistance by biofilms is not fully understood, a number of factors that contribute to this phenomenon depend on the type of bacterium and the type of antibiotic treatment (Hall–Stoodley and Stoodley, 2009). The development of gradients within biofilm clusters has been found to create anoxic, acidic and nutrient–depleted zones which can activate a dormancy state responsible for generalised resistance of biofilms to antibiotics (Stoodley *et al.*, 2008; Walters *et al.*, 2003). The inability of potent antibiotics even at high concentrations to completely inhibit or eradicate biofilms is possibly partly due to the presence of persister cells (Lewis, 2007).

Persister cells are the sub-population of bacteria that are found to be produced during stationary growth phase or become tolerant to antimicrobial killing and survive. They are responsible for repopulating and continuing biofilm infections through dispersal and colonisation of new niches (Lewis, 2001; Lewis, 2007; Keren *et al.*, 2004; Keren *et al.*, 2012). Persister cells account for approximately 1% of the total biofilm population (Anderson and O’Toole, 2008; Hall–Stoodley and Stoodley, 2009; Lewis, 2001; Lewis, 2007). Though the mechanisms underlying the formation of persister cells remain a puzzle, it is known that the highest rate of persister formation is at stationary phase of growth and independent of quorum sensing as spent growth media and early exponential cultures of *E. coli* or *P. aeruginosa* added together did not show appreciable increase in the number of persister cells isolated (Lewis, 2007). In this study, the ability of *K. pneumoniae* and *P. mirabilis* to produce persister cells from planktonic and biofilm cultures after exposure to antibiotics such as ceftazidime and levofloxacin was assessed. The production of persister cells by the two clinical strains (*K. pneumoniae* and *P. mirabilis*) was compared with the dynamics of persister cell production by 3 well studied strains namely; *E. coli*, *P. aeruginosa* and *S. aureus* (Keren *et al.*, 2004; Keren *et al.*, 2012; Lewis, 2000; Spoering and Lewis, 2001). In the present study, persister cells produced by DFU isolates were tested to determine their survival after exposure to increased concentrations of CAZ and LEV. CAZ, like any other  $\beta$ -lactam antibiotic disrupts peptidoglycan layer synthesis by inhibiting transpeptidases responsible for the crosslinking of the peptidoglycan layer during the exponential growth phase. LEV like any other fluoroquinolone can kill slow growing cells during the stationary growth phase (Keren *et al.*, 2004). It was observed that, *K. pneumoniae* and *P. mirabilis* persister cells were tolerant to killing even after 4 hours of exposure to antibiotics

concentrations 25 times the MIC (Figures 4.6). However, CAZ-induced *P. mirabilis* persister cells were undetectable during lag and early exponential phases. This scenario presents with one of two possible outcomes. The first is the possibility of complete eradication of *P. mirabilis* persister cells by CAZ at a concentration high enough to even kill strains with resistance gene determinants (Geller *et al.*, 2011; Keren *et al.*, 2012). The second possibility is the complete shutdown of all metabolic processes and the presence of undetectable levels of persister cells that would resume after the return of favourable growth conditions.

The pattern of *K. pneumoniae* and *P. mirabilis* persister cell production in the growth-state dependent assay was intriguing when antibiotic concentration was doubled (200 µg/mL). Persister cell production at the lag and early exponential phases (Figure 4.7A and C) was similar to the time-dependent killing assay by CAZ in Figure 4.6B. However, persister cells which were undetectable during the lag and early exponential phases were detectable again from the mid-exponential phase, reached high levels at early stationary phase and stabilised throughout the decline phase (Figure 4.7A and C). A similar pattern of *K. pneumoniae* persister cells production was observed when the cells were challenged 3 hours post-incubation (Figure 4.7C) except in Figure 4.7D where the level of CAZ-induced *P. mirabilis* persister cells were detectable and stabilised throughout the growth phase. Again, it can be suggested that the concentrations of CAZ and LEV against the actively dividing *K. pneumoniae* and *P. mirabilis* cells were too high hence the switch from exponential growth to dormancy. In a similar assay, Keren *et al.* (2004) challenged *P. aeruginosa* and *S. aureus* with ciprofloxacin and ofloxacin respectively to monitor the levels of persister cells production in a growing culture. In their work, persister cell production showed a sharp decline during the lag and early exponential phases which was followed by increases in the early to mid-exponential phases and finally stabilised in early stationary phase. In the present study, the dynamics of persister cells formation from *K. pneumoniae* and *P. mirabilis* planktonic cells were different from that reported by Keren *et al.* (2004) and Spoering and Lewis, 2001). The levels of persister cells produced by *K. pneumoniae* and *P. mirabilis* reached high levels at mid-exponential phase and then stabilised through late-exponential, stationary to the decline phases.

Spoering and Lewis (2001) and Keren *et al.* (2004b) have proposed that, the idea that biofilms are resistant to antibiotics than planktonic cells is a misconception and that; persister

cells are fundamentally responsible for biofilm resistance to killing by antibiotics and not by the presence of biofilm specific resistance mechanisms. Although biofilms are largely protected by the EPS barrier from disruption, majority of the embedded cells are susceptible to killing by antibiotics (Spoering *et al.*, 2001). They also reported that, biofilm and stationary-phase cultures of *P. aeruginosa* were tolerant to tobramycin and noted that at low antibiotic concentrations, persister cells were revived to repopulate the biofilm. In the present study, the killing pattern of *K. pneumoniae* and *P. mirabilis* biofilms by LEV was found to be similar to that of their planktonic counterparts (Figure 4.6). Unlike what was reported by Spoering and Lewis (2001), the levels of persister cells produced from biofilms in the present study were rather higher than their counterparts produced from planktonic cells (Figures 4.6 and 4.8). The present study also confirms that persister cells productions are growth-phase dependent with high levels detectable in late-exponential and early stationary phases.

Attempts were made to find differences between *K. pneumoniae* and *P. mirabilis* persister cells and the wild-type. Three assays were performed to consider the fact that, persister cells are not genetic mutants of the wild-type. Biofilm-derived persister cells and the planktonic counterparts were found to have the same antibiotic susceptibility profiles when tested against ampicillin, ceftazidime, ciprofloxacin, gentamicin and levofloxacin. A time-dependent assay showing the production of *K. pneumoniae* and *P. mirabilis* persister cells after challenged with LEV showed the same killing pattern observed with the wild-type. A similar observation was made by Keren *et al.* (2004). It has been argued that the blocking of the DNA replication fork by the DNA gyrase-fluoroquinolone complex during bacterial replication is a temporary process that cannot induce defects in bacterial cells leading to persister cell formation (Keren *et al.*, 2004). This idea was revisited in the present work to determine whether levofloxacin, whose primary target is DNA gyrase (Hooper, 2000), is likely to induce any defect that results in persister cell formation. However, gel electrophoresis and DNA sequencing following PCR amplification of the gyrase B gene in both *K. pneumoniae* and *P. mirabilis* persister cells and the wild-type showed no differences between them. These findings suggest that *K. pneumoniae* and *P. mirabilis* persister cells are not genetic variants and hence identical to their wild-type counterparts. Currently, the only bacterial model that has been found to genetically regulate persistence is in *E. coli* through the toxin-antitoxin (TA) system (Moyed and Bertrand, 1983).

The *Quasi-Vivo*® assay is a continuous flow system adapted in the present work to mimic *in vivo* settings to study the effect of antibiotics (CAZ and LEV) on established biofilm. The degree of biofilm eradication was monitored in a time-dependent assay that illustrated a direct relationship between biofilm eradication and time for the first few hours of the assay. Interestingly, the concentrations (512 and 5120 µg/mL) of CAZ and LEV used in this assay showed little or no difference in the degree of biofilm eradication showing a similar pattern of killing previously observed (Figure 4.9). The results presented here also indicate that persister cells production is not only an antibiotic-specific phenomenon but also specialised cells that are produced at low levels in stationary phase or biofilm cultures and regulated by the bacterial population throughout its growth cycle (Keren *et al.*, 2004).

In summary, it can be said that the colonisation of diabetic foot ulcers by *K. pneumoniae* and *P. mirabilis* may result in the establishment of biofilm infections in DFUs that may be difficult to treat by antibiotics. Non-healing DFUs with accompanying biofilm infections which do not respond to antimicrobial treatment may increase the risk of amputations in DFU patients. The risk of amputation among DFU patients with established biofilm infections may also be aggravated by the presence of persister cells which have been found to be tolerant to antimicrobial killing. Although high concentrations of antibiotics (i.e., above recommended therapeutic levels) have been found (including this study) to significantly inhibit and/or eradicate biofilms, their administration would induce tissue damage or cytotoxicity. In order to decrease these high antibiotic concentrations to achieve therapeutic levels, this study further proposes that the formulation of a topical treatment option that will combine the efficacies of two or more antimicrobial agents at either sub-inhibitory or minimum inhibitory concentrations may facilitate wound healing.

## Chapter 5

Effects of antibiotics and anti-biofilm agents on quorum sensing and cell surface interactions in biofilm-forming DFU isolates

## 5.1 Introduction

Quorum sensing (QS), also called bacterial communication, is a process where bacteria in a community produce, release, detect and respond to small chemical signalling molecules called autoinducers that correspond to a threshold of cell population density (Davies *et al.*, 1998; Miller and Bassler, 2001; Ng and Bassler, 2009; Parsek and Greenberg, 2005; Schauder and Bassler, 2001; Waters and Bassler, 2005; Whitehead *et al.*, 2001). Through quorum sensing bacteria can simultaneously regulate gene expression in response to changes in cell density and species variation (Ng and Bassler, 2009). This synchronised behaviour has been linked to regulation of cellular processes such as bioluminescence, antibiotic production, antibiotic resistance, biofilm formation and virulence expression (Davies *et al.*, 1998; Engebrecht and Silverman, 1984; Haas *et al.*, 2002; Ng and Bassler, 2009). The two most commonly described QS systems are the acyl-homoserine lactone (acyl-HSL) signalling system in Gram-negative species and the autoinducing peptide signalling system in Gram-positive species (Bassler, 2002; Sturme *et al.*, 2002). A third system called the autoinducer-2 QS system, which was first described in *Vibrio harveyi* and implicated in interspecies communication has been found to be produced by a large number of Gram-negative and Gram-positive species (DeKeersmaecker and Vanderleyden, 2003; Surette and Bassler, 1998). The signalling molecule for the AI-2 QS systems is a furanosyl borate diester (Chen *et al.*, 2002).

Bacterial communication in Gram-negative species was first discovered in the marine bioluminescent bacterium *Vibrio fischeri* (Hastings and Nealson 1977). The typical Gram-negative QS system is made of the LuxI/LuxR (HSL/transcriptional activator) signalling circuits (Fuqua *et al.*, 1995; Miller and Bassler, 2001). However, QS in *V. harveyi*, another bioluminescent bacterium, does not use the typical LuxI/LuxR signalling system but rather an evolved QS system with characteristics that resemble both Gram-negative and Gram-positive QS systems (Schauder and Bassler, 2001). *V. harveyi* makes and responds to 3 autoinducers (*V. harveyi* autoinducer 1 (HAI-1), *V. cholerae* autoinducer 1 (CAI-1) and AI-2) in three parallel QS systems that eventually controls the production of the master regulator LuxR (Henke and Bassler, 2004a; Henke and Bassler 2004b; Waters and Bassler, 2006). The production of LuxR has been found to directly or indirectly control the expression of genes required for bioluminescence, biofilm formation, type III secretion and protease production

(Waters and Bassler, 2006). It has been suggested that AI-2 and CAI-1 maybe associated with *V. harveyi* biofilms as mixed *Vibrio* consortium would contain significant levels of both AI-2 and CAI-1 (Waters and Bassler, 2006). It was observed that AI-2 consumptions in mixed *V. harveyi* – *E. coli* cultures significantly altered QS regulation in *V. harveyi* (Xavier and Bassler, 2005a; Xavier and Bassler, 2005b). Xavier and Bassler (2005b) therefore suggested that other bacteria with AI-2 QS systems could coexist with *V. harveyi* in *in vivo* settings such as in the gastrointestinal tracts of marine animals. In *P. aeruginosa*, there is a hierarchical QS system; two LuxI/LuxR pairs (LasI/LasR and RhlI/RhlR) that function together to control virulence expression and biofilm formation (Brint and Ohman, 1995; Parsek and Greenberg, 1999). A specific *Pseudomonas* quinolone signal (PQS) whose biosynthesis and function is mediated by the *las* and *rhl* QS systems is responsible for the control of *lasB* gene which encodes for the LasB elastase virulence factor (Calfee *et al.*, 2001). A third regulator, called the quorum-sensing-controlled regulator encoded by the *qscR* gene and homologous to the LasR and RhlR signal receptors has also been found to repress *lasI* which regulates acyl-HSL production (Chugani *et al.*, 2000). *Agrobacterium tumefaciens*, like *V. fischeri*, also has the Ti plasmid TraI/TraR QS signalling circuit that activates and regulates the expression of genes responsible for mating between bacterial strains and the mobilisation of virulence factors in the Ti plasmid (Fuqua *et al.*, 1995; Zhang *et al.*, 1993). Reference *A. tumefaciens* strains KYC6, A136 and NTL4 have widely been used as biosensor reporter strains for the production and detection of the different AHL molecules produced by Gram-negative bacteria in bacterial cultures (Rickard *et al.*, 2010; Stickler *et al.*, 1998; Yin *et al.*, 2012). *A. tumefaciens* biosensor strains carry the *traI-lacZ* reporter fusion gene which is activated by a transcriptional activator protein in the presence of AHL molecules (Stickler *et al.*, 1998). The transcription of the fusion gene results in the production of  $\beta$ -galactosidase which cleaves X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside), a  $\beta$ -galactosidase substrate, in a reaction that results in a distinctive blue pigmentation of colonies on agar plates (Stickler *et al.*, 1998; Yin *et al.*, 2012).

In bacterial ecological units, the ability to rapidly colonise a surface and form biofilm is central to their survival in the midst of other competitors (Verstraeten *et al.*, 2008). Costerton *et al.* (1987) and Brown and Williams (1985) have suggested that the adherence of bacteria to both biological (such as tissues) and non-biological (environmental) surfaces and to each other during biofilm formation and colonisation of niches may be promoted by the synthesis

of cell–surface associated polysaccharides called capsular polysaccharides (CPS). As a result, Jenkinson (1994) suggested that the interaction of bacterial (mono–species or multispecies) consortia within a biofilm may partly be mediated through glycan–lectin interactions that involve capsular polysaccharides and other ligands. In addition, capsular polysaccharides have been found to protect the growing biofilm from host–immune defence mechanisms such as lactoferrin, macrophages and complement 3 (Kamiya *et al.*, 2012). The CPS acts as a thick matrix that prevents host immune attack (Schembri *et al.*, 2005). Bacterial cell–surface appendages such as fimbriae have also been found to be expressed and associated with biofilm formation during initial adhesion (Schembri *et al.*, 2005). Gram–negative fimbrial adhesins such as types 1 and 3 found at the tip of most fimbriae have been demonstrated to have specificity in binding to carbohydrate–containing receptors on the surfaces of others cells during cellular adhesion (Gygi *et al.*, 1995; Schembri *et al.*, 2005; Schroll *et al.*, 2010). Though the long fimbrial structures allow them to penetrate the CPS, the CPS can interfere with their adhesive properties (Goncalves *et al.*, 2014; Schembri *et al.*, 2005).

It has been demonstrated that, the expression and functions of the CPS and fimbriae may be regulated by quorum sensing (Chemani *et al.*, 2009; Schembri *et al.*, 2005; Tuson, and Weibel, 2013). For example, the role of LecA and LecB lectins (carbohydrate–binding proteins) which specifically bind to D–galactose and D–fucose in *P. aeruginosa* pathogenesis and biofilm formation in lung infection has been found to be regulated by quorum sensing (Chemani *et al.*, 2009; Diggle *et al.*, 2006; Tielker *et al.*, 2005; Winzer *et al.*, 2000). A third group of polysaccharides associated with biofilm formation is synthesised as part of the EPS by bacteria in a biofilm consortium. These polysaccharides which primarily make up the bulk of the EPS, together with other synthesised biopolymers such as extracellular DNA, proteins and lipids, make up the intercellular space of biofilm aggregates facilitating the differentiation of the growing biofilm (Vu *et al.*, 2009). The main function of the EPS is to provide structural support to the mature biofilm (Branda *et al.*, 2005; Latasa *et al.*, 2006; Lopez *et al.*, 2010; Rice *et al.*, 2007). Extracellular polysaccharides, also called exopolysaccharides produced by some biofilm–forming bacterial strains include alginate, glucose–rich PEL and mannose–rich PSL by *P. aeruginosa*; polysaccharide intercellular adhesin (PIA) and poly–N–acetylglucosamine (PNAG) by *S. aureus*; and poly– $\delta$ –glutamate (PGA) and the polysaccharide EPS by *Bacillus subtilis* (Branda *et al.*, 2006). EPS production



by biofilm-forming bacteria has been found to be regulated by quorum sensing (Davies *et al.*, 1998; Donlan, 2002; Ruiz *et al.*, 2008; Waters and Bassler, 2005).

The characterisation of the carbohydrate contents of the capsular polysaccharide, exopolysaccharides and other cell-surface glycans involved in adhesion and biofilm formation has been made possible through assays such as gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), high pressure anion exchange chromatography (HPAEC) and electron microscopy (Al-Halbouni *et al.*, 2009; Goncalves *et al.*, 2014; Pierre *et al.*, 2012; Wozniak *et al.*, 2003). The carbohydrate contents of some strains of *P. aeruginosa* include mannose, glucose, galactose, mannuronic acid, rhamnose, N-acetyl fucosamine, N-acetyl galactosamine and N-acetyl glucosamine (Wozniak *et al.*, 2003). Cell-free extracts of *K. pneumoniae* containing CPS and analysed by HPAEC were found to be composed of monosaccharides such as galactose, glucose, rhamnose, glucuronic acid and glucosamine (Goncalves *et al.*, 2014). Gygi *et al.* (1995) also demonstrated through GC-MS that *P. mirabilis* cell-surface polysaccharides were composed of galacturonic acid and galactosamine. However, characterisation of the carbohydrate contents of bacterial cell-surface polysaccharides using the above-mentioned methods is expensive and is mostly beneficial for research purposes. The characterisation of the carbohydrate contents of biofilm-associated exopolysaccharides and bacterial cell-surface polysaccharides may prove to be new targets in the strategic development and design of effective anti-biofilm therapeutic agents (Bales *et al.*, 2013; Vu *et al.*, 2009).

The individual use of biocides, antibiotics, antimicrobial peptides (such as polymyxin B and dermicidin) and antimicrobial wound dressings in the treatment of biofilm-infected chronic wounds as single treatment regimen has proven futile (Cowan, 2011; Hill *et al.*, 2010; Percival *et al.*, 2007). The biofilm EPS in addition to its role as a structural support for the biofilm also acts as a diffusion barrier that prevents the entry of large antimicrobial peptides/proteins such as lactoferrin, lysozyme and complement from the host immune system as well as antibiotics, thereby conferring resistance to the biofilm (Bjarnsholt *et al.*, 2005; Kimaya *et al.*, 2012; Lewis, 2001). In Gram-negative bacteria such as *P. aeruginosa*, the anionic properties of neutral or polyanionic polysaccharides in the EPS such as cyclic and periplasmic glucans are enhanced by the presence of uronic acids and ketal-linked pyruvate

(Vu *et al.*, 2009). Cyclic and periplasmic glucans bind to divalent cations such as calcium and magnesium to increase the binding force of the mature biofilm (Vu *et al.*, 2009). In addition, the anionic properties of cyclic and periplasmic glucans enable them to bind and sequester aminoglycosides such as gentamicin, kanamycin and tobramycin on the cell surfaces or in the periplasm (Bagge *et al.*, 2004; Brodgen, 2005; Colvin *et al.*, 2011; Mah *et al.*, 2003; Sadovskaya *et al.*, 2010; Shigeta *et al.*, 1997). Mah *et al.* (2003) further suggested that, bacteria embedded within biofilms also employ diverse mechanisms to resist antimicrobial actions. Bacterial resistance mechanisms that are evident during biofilm formation include; target modification by mutation; enzymatic changes leading to target modification; substitution of target (expression of alternative target); modification or destruction of antibiotic; antibiotic efflux, restricted entry of antibiotics and persister cell formation (Lewis, 2007; Mah *et al.*, 2003; Soto, 2013). The role of persister cells in biofilm resistance to antibiotics has been discussed in section 4.8 in Chapter 4. Also, poor efficacy of oral administration of antibiotics against wound biofilms even at high concentrations may probably be due to oxygen limitation (ischaemia), poor tissue perfusion and low metabolic activity rather than poor antibiotic penetration (Lipsky, 2004; Walters *et al.*, 2003). The penetration of antibiotics or antimicrobial peptides such as polymyxin B and its derivatives may further be exacerbated in diabetic foot ulcer patients with peripheral vascular disease, neuropathy and/or Charcot disease (Hill *et al.*, 2010; Kropec and Daschner, 1991; Martin *et al.*, 2015). Hill *et al.* (2010) further noted that biocides such as iodine impregnated in wound dressings with a gel base did not only have prolonged efficacy against wound biofilms, but also helped to degrade the biofilm EPS. Wound dressings impregnated with silver nanoparticles, Manuka honey, triglycerides, metals such as copper, zinc, gold and iron salts with different concentrations and drug delivery systems such as hydrogels, microemulsions, liposomes and metal nanoparticles have been tested on chronic wounds to determine their efficacies on both growing and mature biofilms (Hill *et al.*, 2010; Martin *et al.*, 2015; Percival *et al.*, 2007). Though these antibiotics/antimicrobial agents, through their delivery systems can be delivered to biofilm-forming bacteria or biofilms, and interfere with mechanisms underlying biofilm formation such as quorum sensing and persister cell formation, they have failed to completely inhibit or eradicate biofilms.

## 5.2 Aims and objectives

The regulation of cellular processes underlying biofilm formation by QS resulting in resistance to clinically relevant antimicrobials and antibiotics is one of the biggest dilemmas as far as treatment of biofilm-related infections are concerned (Beceiro *et al.*, 2013; Drenkard and Ausubel, 2002; Yao *et al.*, 2006; Yi and Tian, 2012). The inhibition of quorum sensing has been considered as an alternative strategy to antibiotic treatment that can be useful in the treatment of chronic infections through the prevention and disruption of biofilm formation. Quorum sensing inhibitors (QSI) such as cinnamaldehyde (CIN), baicalin hydrate (BH) hamamelitannin and furanone derivatives have been found to influence EPS production and biofilm formation a fundamental step in the development of anti-biofilm strategies (Brackman *et al.*, 2011; Estephane *et al.*, 2008; Ponnusamy *et al.*, 2010; Ren *et al.*, 2001). However, the influence of QS in the formation of biofilms in *K. pneumoniae* and *P. mirabilis* is less known compared to other Gram-negative species such as *E. coli* and *P. aeruginosa* which are widely used as model strains for biofilms and QS studies (Lopez *et al.*, 2010).

The aim of this chapter is to investigate molecular mechanisms such as quorum sensing and bacterial cell-surface interactions in biofilm formation by *K. pneumoniae* and *P. mirabilis*. *In silico* techniques, such as bioinformatics database analysis using BioCyc, National Center for Biotechnology Information (NCBI) and Universal Protein Resource (UniProt), as well as *in vitro* assays were employed to study the biosynthetic pathways of QS genes in *Klebsiella pneumoniae*, *Proteus mirabilis* and *P. aeruginosa* clinical strains for autoinducer (AI-1 and AI-2) production. The 3 autoinducers produced by *V. harveyi* have been found to act synergistically to produce light in *V. harveyi* (Henke and Bassler, 2004b). The deletion of any of *V. harveyi* autoinducer synthases (CqsA<sub>vh</sub> for CAI-1; LuxM for HAI-1; and LuxS for AI-2) has been found to significantly reduce light production by 83, 99.9 and 96% respectively in mutant strains (Henke and Bassler, 2004b). However, the addition of exogenous HA-1 and AI-2 signalling molecules restored light production in *luxM* and *luxS* mutants (Bassler *et al.*, 1993; Bassler *et al.*, 1994).

Against the backdrop of light production in *V. harveyi*, the current study investigated the bioluminescence of reference *V. harveyi* mutant strains through the exogenous addition of AI-2 signalling molecules produced from cell-free culture fluids of *K. pneumoniae* and *P.*

*mirabilis* clinical strains. The ability of exogenous AI-2 to induce biofilm formation in the reference *V. harveyi* strains was also accessed. Bacterial cell-cell adhesion and synthesis of polysaccharides in the EPS during biofilm formation were also investigated using a panel of plant lectins to determine the carbohydrate components of these polysaccharides. The role of AI-2 in *K. pneumoniae* and *P. mirabilis* biofilm formation was investigated through the inhibition of QS using a selection of QS inhibitors. The final part of this chapter considered the synergistic effects of antibiotics and antimicrobial agents such as QS inhibitors, cell membrane permeabilisers (PMB and PMBN) and wound dressings on biofilms in an attempt to identify and recommend a topical formulation for biofilm treatment. The following objectives were considered in an attempt to achieve the above aims;

- Determination of quorum sensing (QS) genes in DFU isolates using *in silico* and *in vitro* methods such as bioinformatics tools and PCR amplification respectively.
- To study the evolutionary relationship between DFU isolates and other speciated strains using QS genes.
- Perform QS inhibition assays using MBEC™ Physiology & Genetics assay.
- Perform bioluminescence assay according to the method described by Bassler *et al*, (1994).
- Perform glycan-lectin assay using the enzyme-linked lectin-sorbent assay (ELLA) to confirm bacterial cell surface interactions during biofilm formation
- To determine the synergistic effects of antibiotics and anti-biofilm agents on biofilms.
- To determine the effects of wound dressings on DFU isolates and their synergy with antibiotics on DFU isolates.

### 5.3 Genomic studies of QS activities in Ghanaian DFU isolates

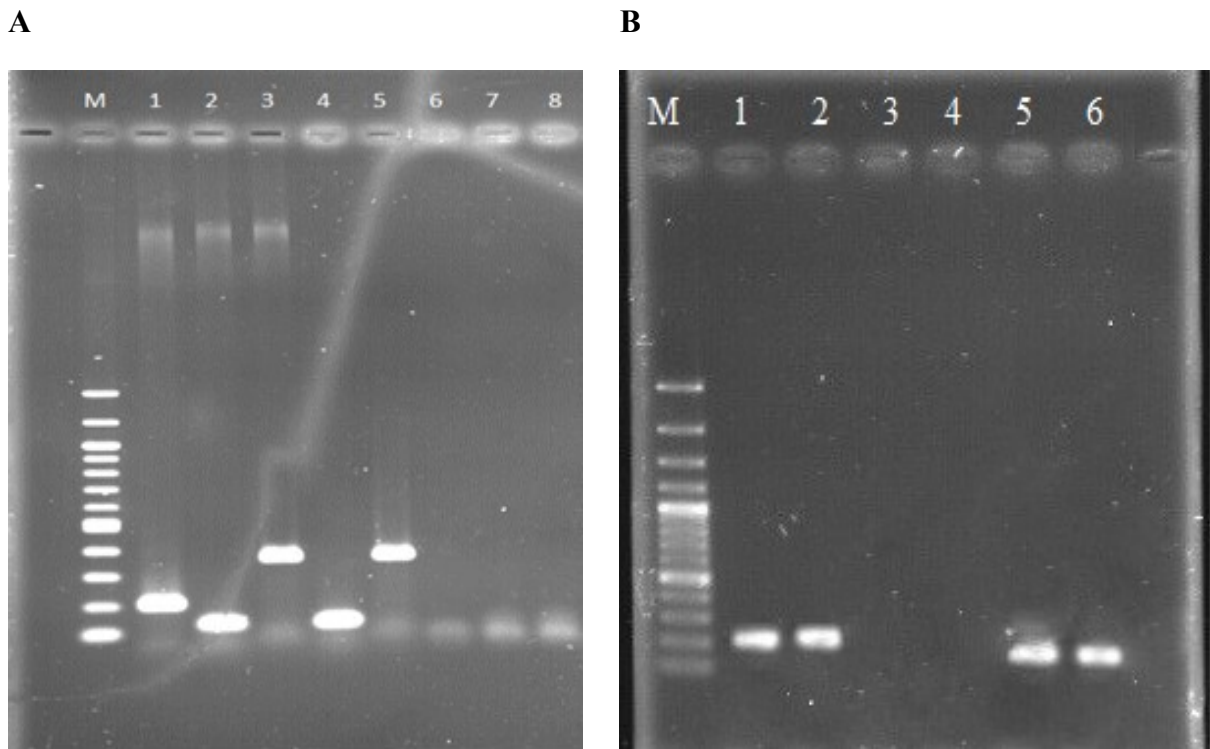
Three strains namely; *Klebsiella pneumoniae* 028b, *Proteus mirabilis* 005 and *P. aeruginosa* 038, (subsequently referred to as *Klebsiella pneumoniae*, *Proteus mirabilis* and *P. aeruginosa*) previously identified (Table 3.7, Chapter 3) were selected as diabetic foot representative strains for these studies. Two reference strains; *P. aeruginosa* PAO1 with *las/rhl* QS systems and *V. harveyi* NCIMB 1280 luminous strain (also known as ATCC 14126) were used as control strains (Pearson *et al.*, 1997; Pesci *et al.*, 1997; Urbanczyk *et al.*, 2008).

#### 5.3.1 *las/rhl* regulated biofilm formation in *P. aeruginosa*

Quorum sensing in *P. aeruginosa* is regulated by 2 hierarchical QS systems designated as *las* and *rhl* (Davies *et al.*, 1998; Juhas *et al.*, 2005; Schuster and Greenberg, 2006; Venturi, 2006). The correspondent increase in the signalling molecule 3-oxo-dodecanoyl (3-O-C<sub>12</sub>) homoserine lactone in response to increase cell density, leads to the formation of signalling molecule-LasR (a transcriptional regulator) complex (Sakuragi and Kolter, 2007). In addition to 315 other QS-activated genes, the 3-oxo-dodecanoyl HSL-LasR complex also directly activates *lasI* (autoinducer-1 synthase promoter gene) and *rhlR* (subordinate transcriptional regulator gene) leading to the regulation of several cellular processes such as virulence and biofilm formation (Gilbert *et al.*, 2009; Sakuragi and Kolter, 2007). It has been found that, *P. aeruginosa* mutants with a defective Las QS system produced amorphous and weak biofilms (Davies *et al.*, 1998). This suggests that Las QS system may be indirectly responsible for the synthesis of exopolysaccharides such as PEL that provide structural support to the growing biofilm (Davies *et al.*, 1998; Friedman and Kolter, 2004; Sakuragi and Kolter, 2007; Vasseur *et al.*, 2005). Since the sequential recognition of LasR and RhlR regulated processes leading to biofilm formation in *P. aeruginosa* is not fully defined, the predictions of such sequential process is difficult (Sakuragi and Kolter, 2007). In the current study, the presence of the *las/rhl* QS system in a DFU *P. aeruginosa* strain was determined by PCR (Figure 5.1A) and the sequenced DNA was compared with other published QS related genes that regulate biofilm formation in *K. pneumoniae* and *P. mirabilis*. BLAST and Genome Browser analyses of NCBI and BioCyc databases respectively failed to detect any QS gene in *K. pneumoniae* and *P. mirabilis* homologous to the *las/rhl* autoinducer-1 QS system in *P. aeruginosa*.

### 5.3.2 Light production in *V. harveyi*

Light production by luminous bacteria (also called bioluminescence) has been previously described by Bassler *et al.* (1993) and Bassler *et al.* (1994). Bioluminescence in *V. harveyi* is synergistically controlled by HAI-1 and AI-2 QS systems (Bassler *et al.*, 1993; Bassler *et al.*, 1994). In the HAI-1 – QS system, HAI-1 (*N*-(3-hydroxybutanoyl) HSL) produced by the autoinducer-1 synthase, LuxM is detected by a sensor kinase, LuxN at high cell density (Bassler *et al.*, 1993). At high cell density, autoinducer-2 synthase, LuxS produces AI-2 which is detected by the AI-2 sensors LuxP and LuxQ (Chen *et al.*, 2002; Surette *et al.*, 1999). At high cell density LuxN and LuxQ switch from being kinases to phosphatases and unphosphorylate and inactivate the response regulator, LuxO leading to the expression of *luxR* which encodes the activator protein LuxR (Freeman *et al.*, 2000; Lenz *et al.*, 2004). LuxR subsequently binds to promoter region and activates the expression of the *luxCDABE* operon encoding the luciferase and fatty acid reductase complex needed for light production (Bassler *et al.*, 1993). For the purpose of the current study, a luminous reference strain *V. harveyi* NCIMB 1280 (also designated as *V. harveyi* ATCC 14126 strain) which is a *luxM* and *luxS* mutant (HAI-1<sup>-</sup> AI-2<sup>-</sup>), and an aldehyde (*luxCDE*) (HAI-1<sup>-</sup> AI-2<sup>-</sup>) mutant reference strain *V. harveyi* NCIMB 1872 (an aldehyde mutant of *V. harveyi* ATCC 33843 (392 [MAV]) were employed for bioluminescence studies (Jensen *et al.*, 1980; Johnson and Shunk, 1936; Nealson and Markovitz, 1970; Wang *et al.*, 2015). The presence of the luciferase gene was determined by PCR (Figure 5.2B).



**Figure 5.1** PCR amplification of partial DNA sequences of; (A) *lasI* (126 bp, in lanes 2 and 4), and *rhlR* (364 bp, in lanes 3 and 5) QS genes in *P. aeruginosa* and *P. aeruginosa* PAO1 respectively. Lanes M – 100 bp DNA ladder, 1 – 203 bp 16S rRNA V3 hypervariable gene in *P. aeruginosa* (positive PCR control) and lane 6 is negative control (B). 190 bp *luxA* (lanes 1 and 2) QS genes in *V. harveyi* reference strains 1280 and 1872. Lanes 3 and 4 – negative controls and, lanes 5 and 6 – 125 bp 16S rRNA V6 hypervariable genes in *V. harveyi* as positive PCR controls.

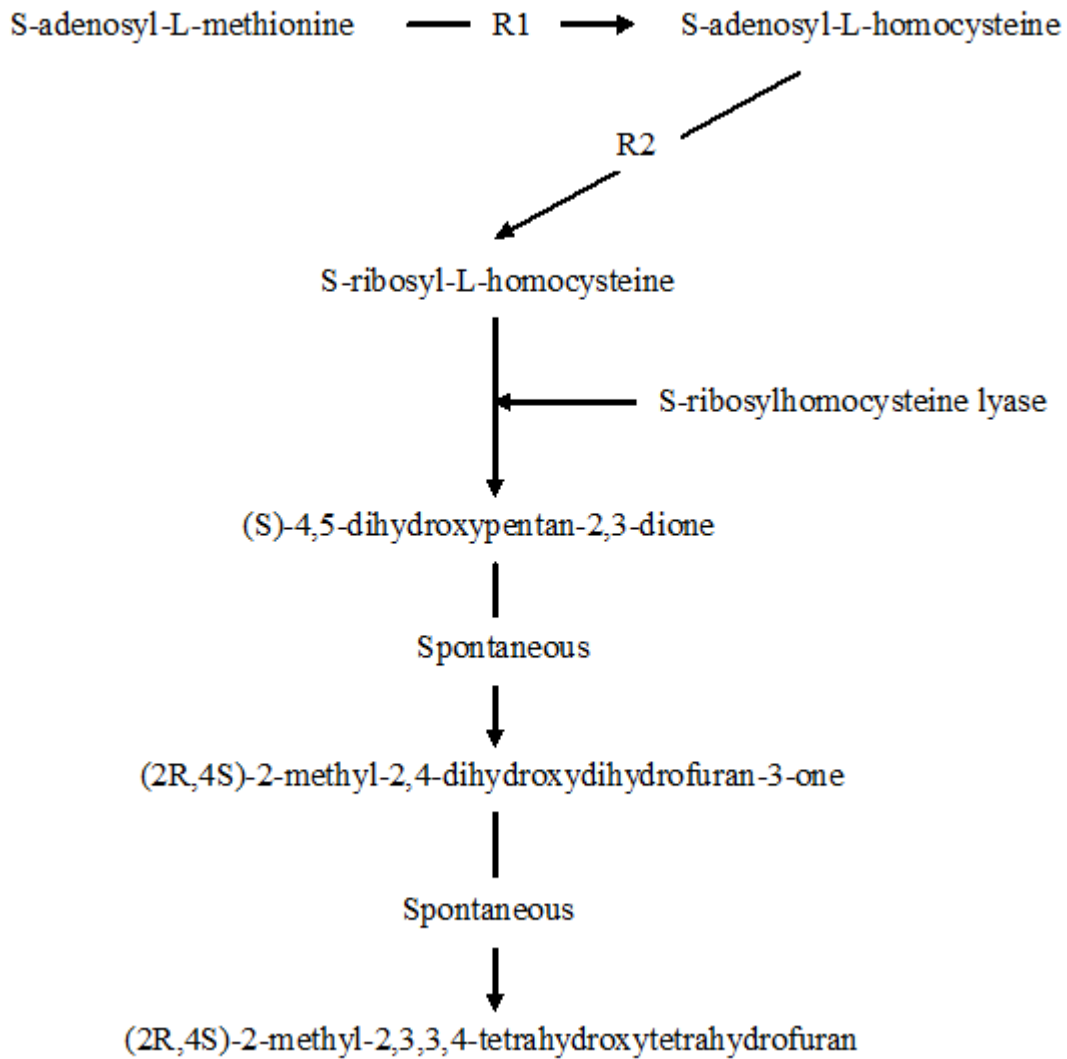
### 5.3.3 AI–2 biosynthesis in *V. harveyi*, *K. pneumoniae* and *P. mirabilis*

Two biosynthetic pathways have been found to be responsible for the production of AI–2 in *V. harveyi* (and other members of the bacterial order *Vibrionales*) and other Gram–negative bacteria (Bassler *et al.*, 1994; Miller *et al.*, 2004; Rezzonico and Duffy, 2008). In both AI–2 biosynthetic pathways, the precursor compound S–adenosyl–L–methionine donates a methyl group to become S–adenosyl–L–homocysteine in a reaction catalysed by S–ribosylhomocysteine lyase (LuxS) with S–ribosyl–L–homocysteine and L–homocysteine as the major end–products (Schauder *et al.*, 2001; Surette *et al.*, 1999; Zhu and Mekalanos, 2003; Zhu *et al.*, 2003; Zhu *et al.*, 2004). The production of the final AI–2 molecule from L–homocysteine which is detected by members of the order *Vibrionales* and other Gram–negative bacteria such as *Salmonella enterica* serovar *typhimurium* creates the difference

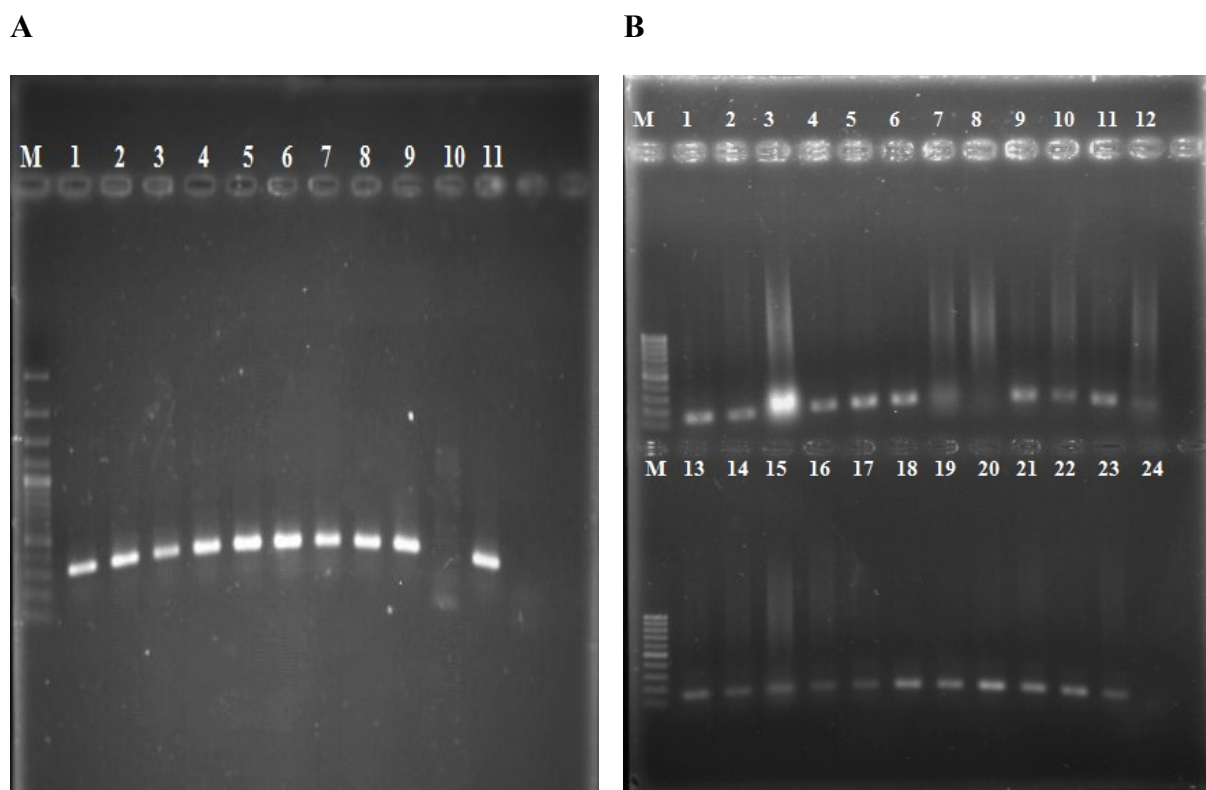
between the 2 biosynthetic pathways. The precise nature and cascade of reactions that result in the ultimate AI-2 production is species specific and involves spontaneous transformations that include cyclisation and isomerisation processes leading to (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran synthesis in *S. enterica* serovar *typhimurium* (as shown in Figure 5.2) and (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate in *Vibrionales* (Bassler *et al.*, 1994; Chen *et al.*, 2002; Miller *et al.*, 2004; Rezzonico and Duffy, 2008). The recognition of AI-2 in *Vibrionales* is by dedicated AI-2 receptors encoded by the *luxP* and *luxQ* genes (Bassler *et al.*, 1994; Reading and Sperandino, 2006). The AI-2 in *S. typhimurium* and other Gram-negative bacteria is recognised by AI-2 receptors belonging to the ABC transporter-periplasmic binding protein family (Miller *et al.*, 2004).

Unlike *K. pneumoniae* and *P. mirabilis*, the presence and processes underlying QS-regulated activities in *V. harveyi* such as virulence and biofilm formation have been well studied (Bassler, 1993; Bassler and Silverman, 1994; DeKeersmaecker and Vanderleyden, 2003; Henke and Bassler, 2004a; Henke and Bassler 2004b; Surette and Bassler, 1998; Waters and Bassler, 2006; Surette and Bassler, 1998; Xavier and Bassler, 2005a; Xavier and Bassler, 2005b). In the current study, BioCyc database was employed in the analysis of biosynthetic pathways leading to the production of AI-2 in *V. harveyi*, *K. pneumoniae* and *P. mirabilis*. The database model strains used in these analyses were *Klebsiella pneumoniae* 342, *Proteus mirabilis* HI4320 and *Vibrio harveyi* CAIM 1792 (Espinoza-Valles *et al.*, 2012; Fouts *et al.*, 2008; Pearson *et al.*, 2008). Biosynthetic pathways leading to AI-2 productions in *K. pneumoniae* and *P. mirabilis* was found to be similar to that previously described in *S. typhimurium* (above) resulting in the production of (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran as shown in Figure 5.2 (Miller *et al.*, 2004; Xavier and Bassler, 2005b). In addition, AI-2 mediated QS in *K. pneumoniae* leads to the expression of the *lsrACDBFGE* operon, cytoplasmic kinase *lsrK* gene and the transcriptional repressor *lsrR* gene (De Araujo *et al.*, 2010; Taga *et al.*, 2003; Xavier and Bassler, 2005b). In this study, primers were designed to amplify the *luxS* gene that encodes the synthesis of LuxS, the main enzyme that catalyses the hydrolysis of S-ribosylhomocysteine to L-homocysteine and autoinducer-2 in *K. pneumoniae* and *P. mirabilis* (Figures 5.3A and B). The *luxS* PCR amplicons of *K. pneumoniae* and *P. mirabilis* were sequenced and the DNA sequences compared to published *luxS* DNA sequence of *V. harveyi* in the databases (section 5.3.4).





**Figure 5.2** Summary of the AI-2 biosynthetic pathways in *K. pneumoniae* 342 and *P. mirabilis* H14320. In reaction 1 (R1), S-adenosyl-L-methionine donates a methyl group to become S-adenosyl-L-homocysteine in a reaction catalysed by a methyltransferase. In reaction 2 (R2) 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase catalyses the hydration of S-adenosyl-L-homocysteine to release an adenine and form S-ribosyl-L-homocysteine (Data adapted with modifications from: <http://biocyc.com/tmp/JP946.gif>).

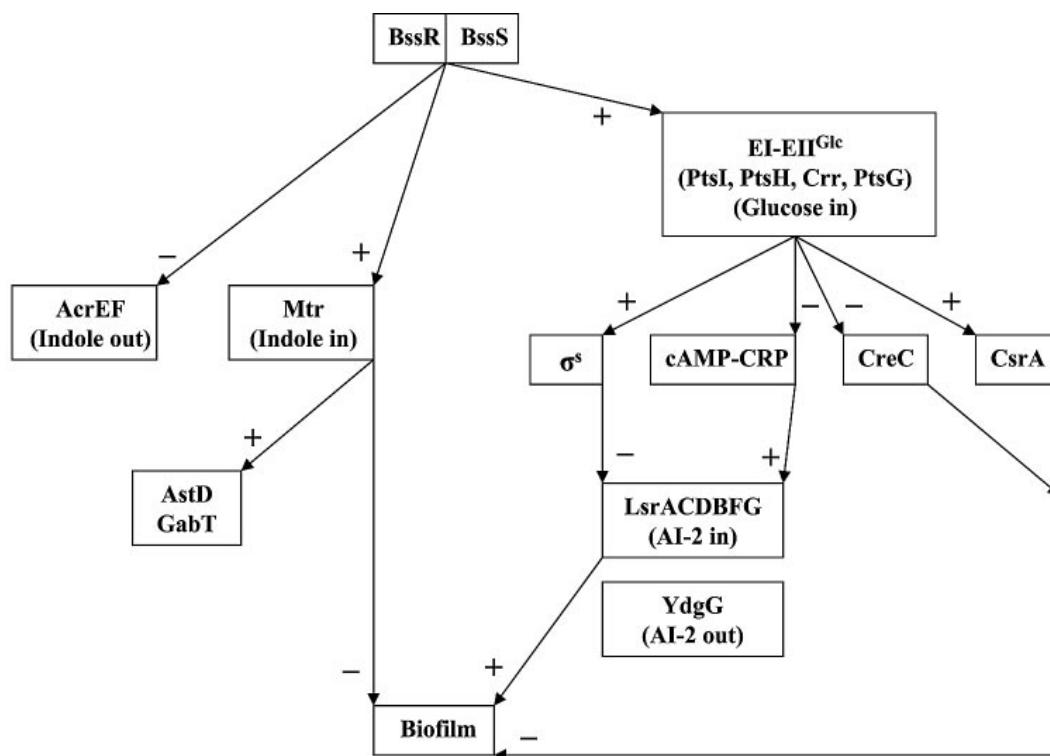


**Figure 5.3** PCR amplification of partial DNA sequence of; (A) 320 bp of the *luxS* QS gene in 9 *K. pneumoniae* (lanes 1 – 10) and 1 *K. variicola* strains (lane 11). Lane 10 – negative control. (B) 152 bp *luxS* QS gene in 22 *P. mirabilis* strains (except in lane 8). Lane 24 – negative control.

#### 5.3.4 Biosynthesis of *lrsB* and *bssS* in *K. pneumoniae* and *P. mirabilis*

At high cell density, the synthesised AI-2 signalling molecules in the extracellular milieu are internalised, phosphorylated, sequestered and destroyed (Taga *et al.*, 2001; Taga *et al.*, 2003; Xavier *et al.*, 2007). The sequestering and destruction of modified AI-2 by some bacteria such as *S. enterica* serovar *typhimurium* has been found to interfere with intercellular communication in a bacterial consortium (Xavier *et al.*, 2007). In the present study, BioCyc Genome Browser analysis tool was used to locate the position of the *lrsACDBFG* operon in the chromosome of *K. pneumoniae* 342 database strain (Appendix A). The presence of the LuxS regulated (Lsr) ABC transporter gene in 10 *Klebsiella* species (9 *K. pneumoniae* and 1 *K. variicola*) was determined by PCR amplification of the *lrsB* gene (Figure 5.5A) which encodes the LsrB ABC transporter – periplasmic AI-2 binding protein responsible for the internalisation of bound AI-2 molecules (Xavier *et al.*, 2007). The PCR amplicons were then sequenced and compared to other *lrsB* genes published in the databases (Figure 5.8).

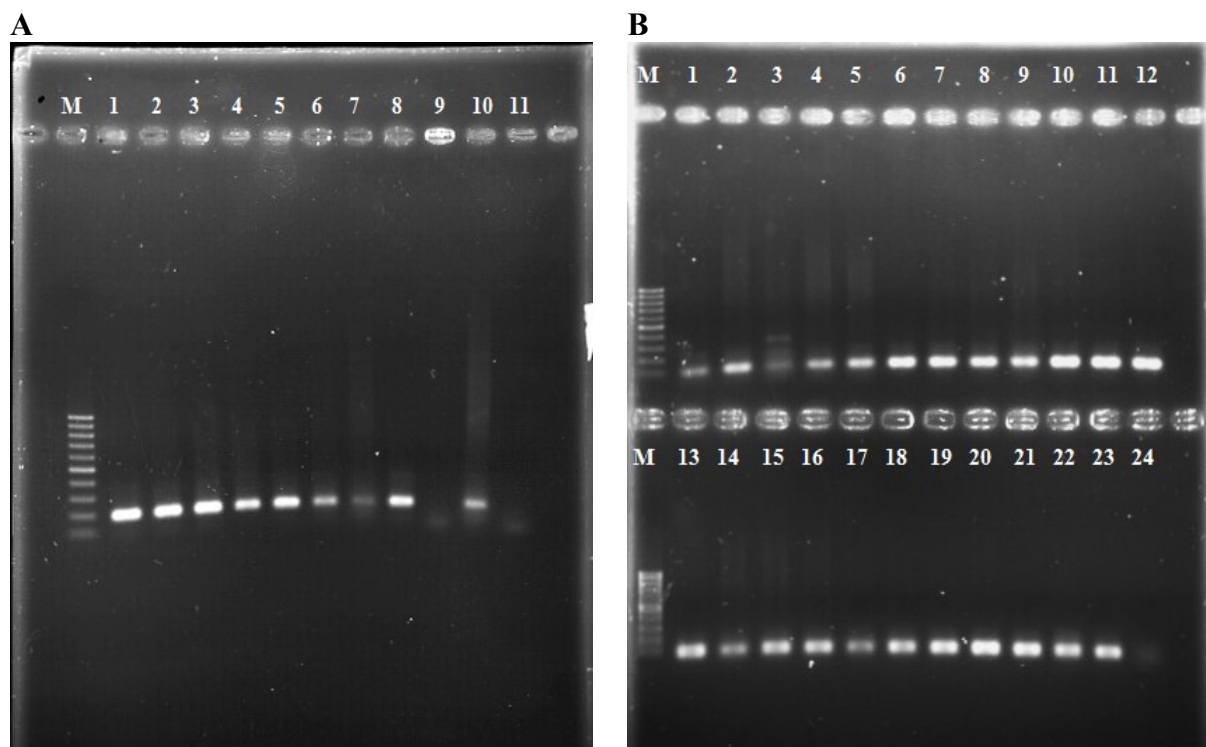
Two biofilm formation regulatory genes known as *bssR* and *bssS* have been found to be induced during biofilm formation in *E. coli* and other Gram–negative bacteria such as *K. pneumoniae*, *P. mirabilis*, *Citrobacter freundii*, *Yersinia pestis*, *Shigella flexneri*, *Salmonella enterica* subspecies *enterica* serovar *typhimurium* and *Enterobacter aerogenes* (Beloin *et al.*, 2004; Domka *et al.*, 2006; Jayaraman and Wood, 2008; Ren *et al.*, 2004; Schembri *et al.*, 2003). The expressions of *bssR* and *bssS* were found to regulate biofilm formation (Figure 5.4) through the uptake and the processing of extracellular AI–2, and the differential expressions of other genes responsible for biofilm formation (Domka *et al.*, 2006).



**Figure 5.4** Proposed regulation (positive (+), negative (–)) of biofilm in *E. coli* by biofilm formation regulatory genes, *bssR* and *bssS* (Domka *et al.*, 2006).

In the current study, the presence of the *bssS* gene in *P. mirabilis* was determined using Genome Browser and Nucleotide/Gene BLAST search tools of BioCyc and NCBI databases respectively and confirmed by PCR amplification of the gene (Figures 5.5B). The *bssS* gene was found to be centrally located (from position 1793752 to 1794006) in the chromosome of *P. mirabilis* HI4320 (Appendix B). Unlike *E. coli*, *K. pneumoniae* and *S. typhimurium* which have both *bssR* and *bssS* genes in the same chromosome, *P. mirabilis* has been found to have only *bssS*. The chromosomal position of the 255 base pairs (bp) *bssS* gene is species–specific.

In the *E. coli* K-12 sub-strain MG1655 chromosome (4,609,408 bp) where *bssR* and *bssS* were first found, *bssS* is located upstream with an intergenic distance of 242070 bp between them. BioCyc Genome Browser analysis further revealed that, while none of the genes is part of an operon, they are also transcribed in opposite directions; with *bssR* towards the right (forward) and *bssS* in the reverse direction. In this study, the partial *bssS* gene sequence amplified by PCR was sequenced and compared with other sequenced *bssS* and *bssR* (384 bp) genes published in the databases (Figures 5.9 and 5.10).

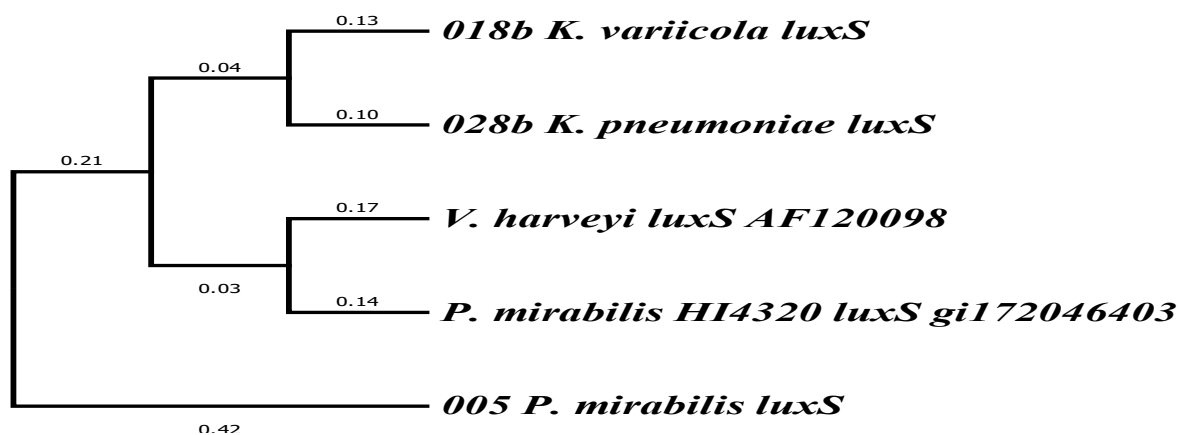


**Figure 5.5** PCR amplification of partial DNA sequences of; (A) 199 bp *lrsB* QS gene in 8 *K. pneumoniae* and 1 *K. variicola* strains (lanes 2 – 9 and lane 10) and lane 11 – negative control, (B) 104 bp *bssS* QS gene in 23 *P. mirabilis* strains. Lane 24 – negative control.

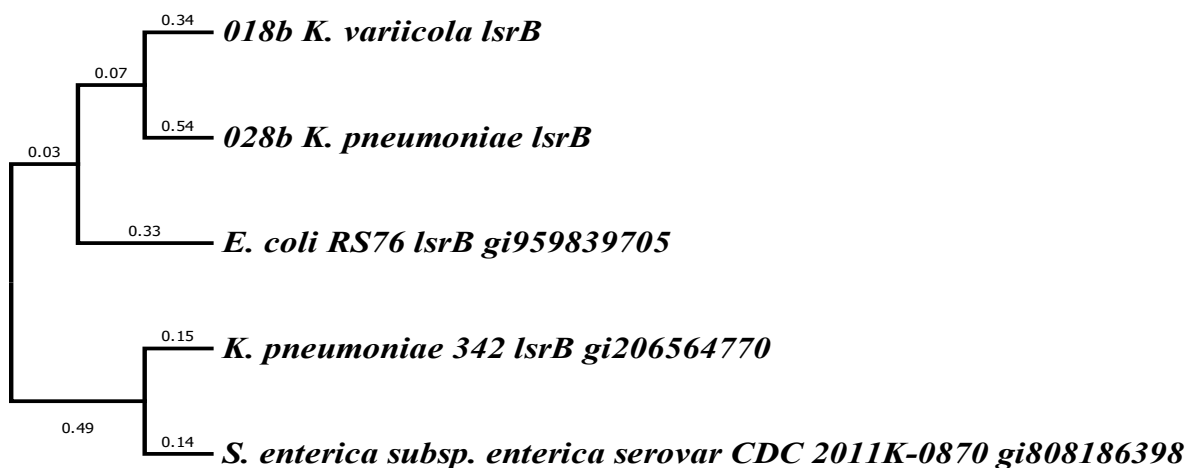
The evolutionary relationships inferred using the cladograms in Figures 5.6, 5.7, 5.8 and 5.9 were based on protein-coding nucleotide sequences for the purpose of homology comparisons. The computer-generated evolutionary distances were for references only and not used for analyses of the trees. Figure 5.6 shows a high degree of homology between the *luxS* genes in 018b *K. variicola* and 028b *K. pneumoniae* as well as between *V. harveyi* (GenBank Accession number AF120098) and *P. mirabilis* HI4320. The *P. mirabilis* strain identified in this study (005 *P. mirabilis*) however showed lower degree of homology to the

others. The multiple sequence alignment in Appendix C used for the construction of the tree provides more details on the homology of all the 5 sequences.

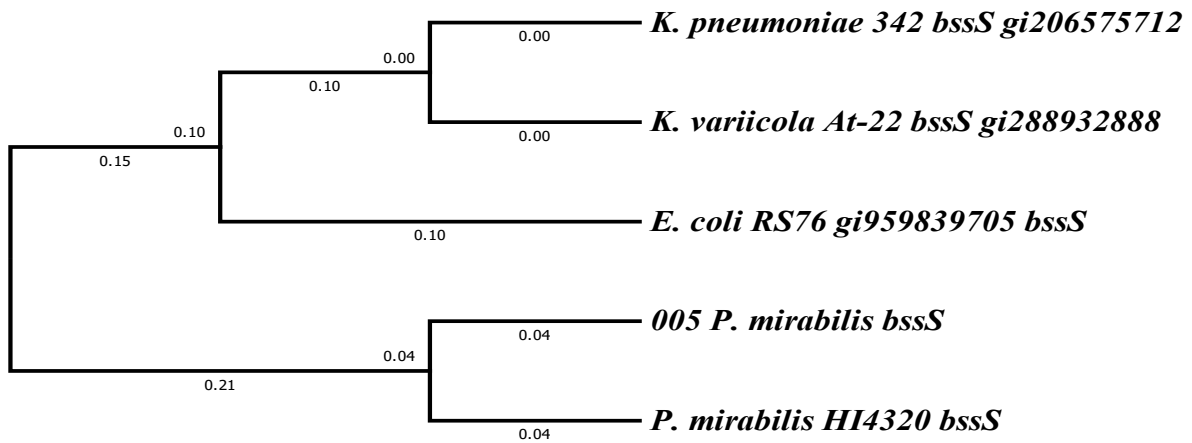
Two *Klebsiella* species identified in this study, 018b *K. variicola* and 028b *K. pneumoniae* showed a high degree of homology between their *lsrB* genes (Figure 5.7). Also, sequence homology between *S. enterica* subspecies *enterica* serovar *typhimurium* CDC 2011K-0870 and *K. pneumoniae* 342 *lsrB* genes was higher than with the *lsrB* gene of *E. coli* RS76 (Figure 5.7, Figure C4 in Appendix C). As expected the *bssS* genes in 005 *P. mirabilis* and *P. mirabilis* HI4320 showed a high degree of homology when aligned together (Figure 5.8). The *bssS* genes in *K. pneumoniae* 342 and *K. variicola* At-22 also showed high degree of homology (Figure 5.8). However, *bssS* gene homology between *E. coli* RS76, *K. pneumoniae* 342 and *K. variicola* At-22 was found to be higher than that between *P. mirabilis* HI4320 and test strain 005 *P. mirabilis* (Figure 5.8). In Figure 5.9, gene sequence homology was found to be higher in the *bssS* clade (005 *P. mirabilis* and *P. mirabilis* HI4320 branches) and the *bssR* clade (*K. pneumoniae* 342 and *S. enterica* subspecies *enterica* serovar *typhimurium* P-stx-12 branches).



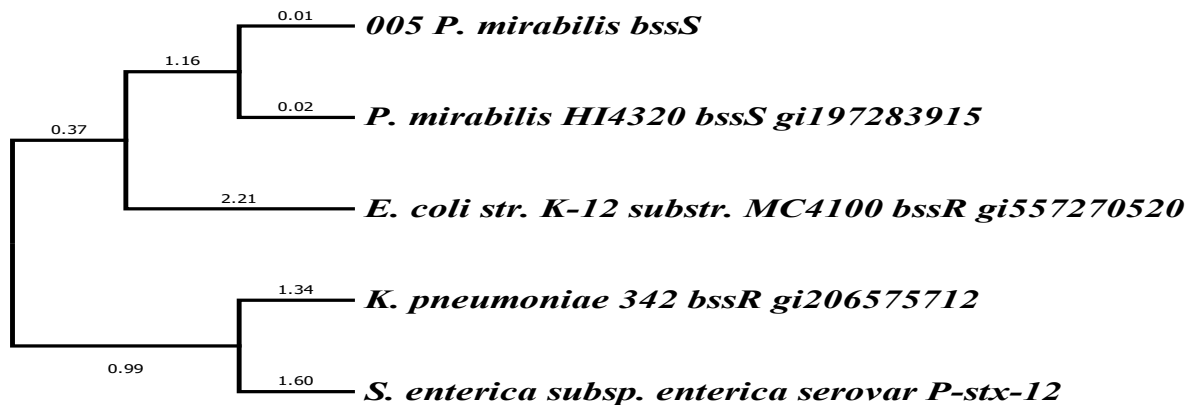
**Figure 5.6** A Cladogram showing the evolutionary relationship between 3 DFU isolates and 2 published speciated strains using the protein-coding nucleotide sequence of the *luxS* gene. Partial DNA sequences for 018b *K. variicola*, 028b *K. pneumoniae* and 005 *P. mirabilis* were used for the analysis. The optimal tree was reconstructed using the Neigbor-Joining (N-J) method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al.*, 2004). All positions containing gaps and missing data were eliminated. There was a total of 124 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).



**Figure 5.7** Cladogram based on *lsrB* gene sequence reconstructed using the N-J method. Five protein-coding nucleotide sequences (partial sequences of 018b *K. variicola* and 028b *K. pneumoniae* from this study; and complete sequences of *K. pneumoniae* 342 *lsrB* gi206564770, *E. coli* RS76 *lsrB* gi959839705 and *S. enterica subsp. enterica* serovar CDC 2011K-0870 gi808186398 from NCBI GenBank) were used to predict their evolutionary relationship.



**Figure 5.8** Cladogram based on the *bssS* gene sequence reconstructed using the N–J method. Five protein–coding nucleotide sequences (partial sequences of 005 *P. mirabilis bssS* gene from this study; and complete sequences of *K. pneumoniae* 342 *bssS* gi206575712, *K. variicola* At–22 *bssS* gi288932888 and *E. coli* RS76 *bssS* gi959839705 from NCBI GenBank) were used to predict their evolutionary relationship.



**Figure 5.9** Cladogram based on the *bssS* and *bssR* gene sequences reconstructed using the N–J method. Five protein–coding nucleotide sequences (partial sequences of 005 *P. mirabilis bssS* gene from this study; and complete sequences of *K. pneumoniae* 342 *bssR* gi206575712, *P. mirabilis* HI4320 *bssS* gi197283915, *S. enterica* subsp. *enterica* serovar *typhimurium* P–stx–12 gi374352002 and *E. coli* str. *K–12* substr. MC4100 *bssR* gi557270520 from NCBI GenBank) were used to predict their evolutionary relationship.

Multiple sequence alignments from which the cladograms were constructed can be referred to from Appendices C2 to C6. Appendix C1 also provides details on the 16S rRNA gene sequence alignment between all the 50 bacterial strains shown in Figure 3.7 in Chapter 3.

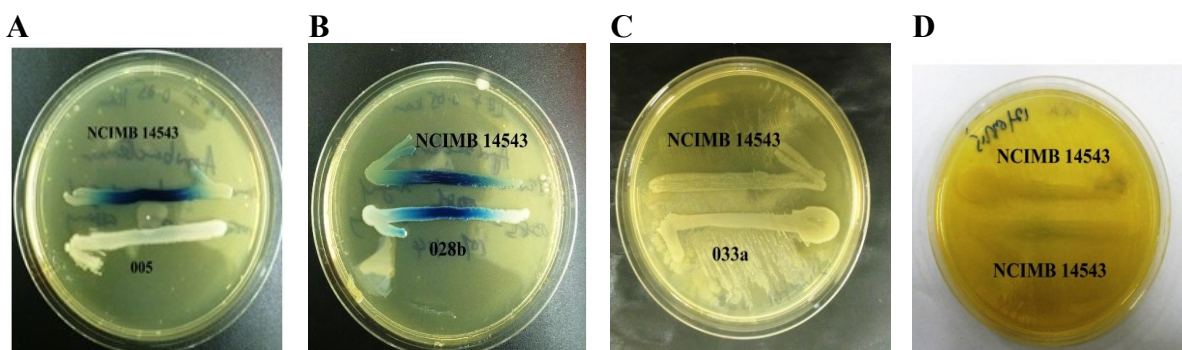
## 5.4 Quorum sensing detection assays

Three QS assays were employed to study QS activities in a selection of DFU isolates. In the biosensor–reporter assay, an *A. tumefaciens* reference strain, NCIMB 14543 with a *lacZ*–fusion reporter gene was used to detect the production of AHL by 37 DFU isolates. The ability of exogenously produced AI–2 from DFU isolates to stimulate the wild–type and mutagenized *V. harveyi* reference strains on sea–water or Luria–marine agar to produce light was also determined. Finally, the ability of the *V. harveyi* reference strains to detect exogenous AI–2 in solution and produce light was quantitatively determined using the bioluminescence assay.

### 5.4.1 Detection of AHL production by DFU isolates

In this study, the *A. tumefaciens* control used in the cross–feeding assay *A. tumefaciens* NCIMB 14543 was a non–AHL producer and a broad–spectrum host that carries a *hyuC*–*lacZ* fusion gene activated by a LuxR family transcriptional regulator in the presence of exogenously produced AHL molecules (Jiwaji, 2006; Jiwaji and Dorrington, 2009; Jiwaji *et al.*, 2008). The production of AHL molecules by the DFU isolates used in this study was detected by the presence of blue pigmentation of the control strain streaked next to the test strain (Figure 5.10A and B). *A. tumefaciens* streaked next to itself (cream colonies) was used as a negative control (Figure 5.10D). Out of the 37 clinical strains tested, 29 (78%) of them were positive for AHL production. Fifteen (65%) out of the 23 *P. mirabilis* strains tested were AHL producers. All 9 *K. pneumoniae* strains as well as the only *K. variicola* strain were found to be AHL producers. The remaining 4 isolates; *C. koseri*, *E. coli*, *P. aeruginosa*, and *P. stuartii* were also positive for AHL production. However, out of the 23 *P. mirabilis* strains 5 of them; 004, 009, 010b, 020 and 037, were slightly positive with a hint of blue pigmentation of the colonies that could just be seen on the agar plates. The remaining 3 *P. mirabilis* strains (014, 032a and 033) were completely negative for AHL production (Table 5.1). All AHL detection assays were repeated at least twice to confirm their reproducibility.



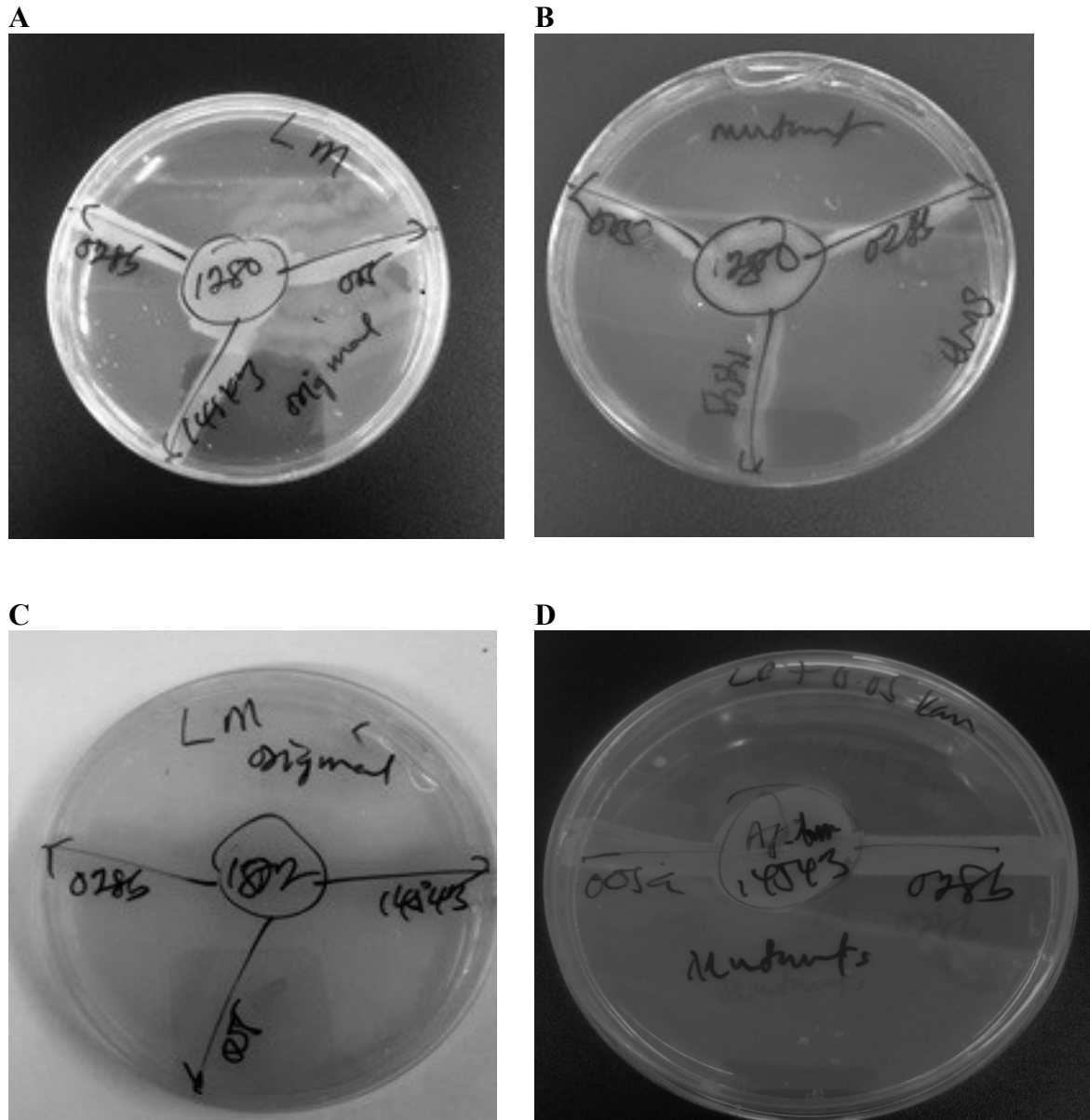


**Figure 5.10** Cross-feeding assay for AHL detection production in DFU isolates using *A. tumefaciens* NCIMB 14543 biosensor reporter strain. *A. tumefaciens* NCIMB 14543 reported AHL production in; (A) 005 – *P. mirabilis*; and (B) 028b *K. pneumoniae*. (C) 033a – *P. mirabilis* showing a negative result and; (D) *A. tumefaciens* NCIMB 14543 against itself as a negative control.

#### 5.4.2 Detection of AI-2 molecules by DFU isolates

In the cross-stimulation assay, *K. pneumoniae* and *P. mirabilis* were used as AI-2 donors to induce bioluminescence in *V. harveyi* reference strains. Three *V. harveyi* strains; wild-type *V. harveyi* NCIMB 1280, mutagenized (dim and dark) *V. harveyi* NCIMB 1280 (designated as *V. harveyi* NCIMB 1280<sub>d</sub>) and an aldehyde mutant *V. harveyi* NCIMB 1872 strain were stimulated by exogenous AI-2 and the intensity of their light production compared (Bolton, 2012). The detection of bioluminescence of the *V. harveyi* strains was done by image capturing in a dedicated darkroom (Figure 5.11A, B, C and D). Bioluminescence was observed at the edges of centrally placed AI-2 recipient *V. harveyi* strains in close proximity to the 028b and 005 (*K. pneumoniae* and *P. mirabilis* respectively) AI-2 donors. The wild-type NCIMB 1280 strain produced the brightest glow on exposure to darkness (Figure 5.11A). The mutagenized NCIMB 1280<sub>dd</sub> also produced a dim glow at the proximal edges indicating reduced functionalities to produce and sustain luminescence (Figure 5.11B). The absence of a functional fatty acid reductase complex in the NCIMB 1872 strain resulted in the absence of aldehyde; one of the substrates oxidised by luciferase to produce fatty acids that leads to light production (Hastings *et al.*, 1985). Reduced functionality of the luciferase enzyme as a result of a defective aldehyde synthesis leads to the formation of aldehyde mutant (dim and dark) strains that produce little or no light even after stimulation by exogenous AI-2 (Hastings *et al.*, 1989; Shimomura *et al.*, 1974). In the present study,

mutagenesis of the wild-type *V. harveyi* NCIMB 1280 strain produced a dim mutant whereas the aldehyde mutant strain *V. harveyi* NCIMB 1872 was confirmed as a dark mutant (Figure 5.11C)



**Figure 5.11** AI-2 cross-stimulation assay for light production in *V. harveyi*. On exposure to darkness; (A) wild-type NCIMB 1280 strain produced the brightest glow followed by, (B) mutagenized NCIMB 1280<sub>d</sub> with dim glow. (C) Aldehyde mutant NCIMB 1872 appeared dark even after 20 mins of exposure to darkness. (D) *A. tumefaciens* NCIMB 14543 was used as the negative control (no light production).

### 5.4.3 Induction of bioluminescence by AI-2 – producing DFU isolates

The AI-2 activity in cell-free supernatants extracted from batch cultures of 23 *P. mirabilis*, 9 *K. pneumoniae* and 1 *K. variicola* strains was determined using a 96-well plate assay. In a separate assay, the synergy between commercially prepared HAI-1, (*N*-(3-hydroxybutanoyl) homoserine lactone) and AI-2 in the bioluminescence of *V. harveyi* was also determined. The GloMax<sup>®</sup>-Multi Detection system (Promega, UK) was used to quantitatively determine bioluminescence, presented as relative light units (RLU) and fold induction of bioluminescence over a period of time. In this study, fold induction of bioluminescence was defined as the ratio of the relative luminescence (in RLU) of the reporter strain (*V. harveyi* NCIMB 1280) cultured in a conditioned AB medium to the that of *V. harveyi* NCIMB 1280 grown in sterile AB medium. A conditioned AB medium (as described in chapter 2, section 2.6.2) is defined as a filter-sterilised cell-free culture supernatant of an organism prepared from an overnight broth culture in AB medium to a predetermined optical density (Schneider *et al.*, 2002) The positive controls were 005 *P. mirabilis* and 028b *K. pneumoniae* (known AI-2 producers) and the negative control was made of AB medium and *V. harveyi* NCIMB 1280 (non-AI-2 producer).

Fold induction of bioluminescence in *V. harveyi* NCIMB 1280 was determined per the method described by Blehert *et al.* (2003) and Rickard *et al.* (2010) with few modifications (Table 5.1). Fold induction of  $\geq 10$  representing 19.3% bioluminescence of *V. harveyi*-*P. mirabilis* positive control were considered positive results as no supplements such as borate was added to increase AI-2 signal strength and increase background signal (Rickard *et al.*, 2010). It is evident in Table 5.1 that all 33 *K. pneumoniae* and *P. mirabilis* DFU strains induced bioluminescence in the *V. harveyi* NCIMB 1280 reporter strain with fold induction  $> 10$ . The 3 highest fold induction values were recorded in 005 *P. mirabilis* (100%), 032a *P. mirabilis* (104%) and 035 *K. pneumoniae* (440%). Although 034a *K. pneumoniae* was negative for *luxS* PCR (Figure 5.3A), it was able induce light production in the *V. harveyi* reporter strain by 35.6%. The concentration of AI-2 produced and detected during batch culture has been found to vary at different time points and differ among chronic wound isolates (Rickard *et al.*, 2010).

**Table 5–1** AHL detection by reporter strain *A. tumefaciens* NCIMB 14543 and induction of bioluminescence in reporter strain *V. harveyi* NCIMB 1280 by cell-free culture supernatants from DFU isolates.

<b>Bacterial strain</b>	<b><i>A. tumefaciens</i> NCIMB 14543</b>	<b><i>V. harveyi</i> NCIMB 1280 (RLU)x10<sup>4</sup></b>	<b>Fold induction of bioluminescence in <i>V.</i> <i>harveyi</i> NCIMB 1280</b>
<i>V. harveyi</i> NCIMB 1872 <sup>1</sup>	ND	ND	ND
001 <i>P. mirabilis</i>	+	40	49.4
002 <i>P. mirabilis</i>	+	38	46.9
003b <i>P. mirabilis</i>	+	30	37.0
004 <i>P. mirabilis</i>	–	30	37.0
005 <i>P. mirabilis</i> *	+	42	51.9
006 <i>P. mirabilis</i>	+	27	33.3
007 <i>P. mirabilis</i>	+	24	29.6
008 <i>P. mirabilis</i>	+	22	27.2
009 <i>P. mirabilis</i>	–	26	32.1
010b <i>P. mirabilis</i>	–	30	37.0
011 <i>P. mirabilis</i>	+	34	42.0
014 <i>P. mirabilis</i>	–	23	28.4
016 <i>P. mirabilis</i>	+	38	46.9
018a <i>P. mirabilis</i>	+	24	29.6
019b <i>P. mirabilis</i>	+	20	24.7
020 <i>P. mirabilis</i>	Slightly +	22	27.2
021a <i>P. mirabilis</i>	+	26	32.1
025b <i>P. mirabilis</i>	+	15	18.5
026 <i>P. mirabilis</i>	+	17	21.0
032a <i>P. mirabilis</i>	–	44	54.3
033 <i>P. mirabilis</i>	–	32	39.5
034b <i>P. mirabilis</i>	+	34	42.0
037 <i>P. mirabilis</i>	Slightly +	29	35.8
018b <i>K. variicola</i>	+	21	25.9
021b <i>K. pneumoniae</i>	+	30	37.0
022b <i>K. pneumoniae</i>	+	18	22.2
023a <i>K. pneumoniae</i>	+	24	29.6
027 <i>K. pneumoniae</i>	+	25	30.9
028b <i>K. pneumoniae</i> *	+	34	42.0
029 <i>K. pneumoniae</i>	+	38	46.9
032b <i>K. pneumoniae</i>	+	39	48.1
034a <i>K. pneumoniae</i>	+	15	18.5
035 <i>K. pneumoniae</i>	+	185	228.4

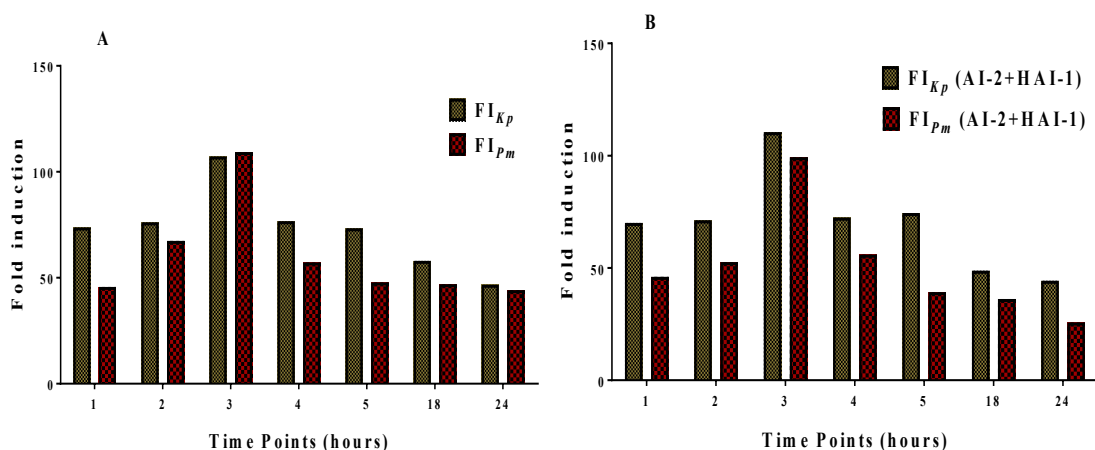
Key for Table 5.1 (above)

(+) and (-) indicate positive and negative results respectively.

<sup>†</sup> - *V. harveyi* NCIMB 1872 – negative control; ND – Not determined.

\* – Positive controls (005 *P. mirabilis* and 028b *K. pneumoniae*). The value for *V. harveyi* NCIMB 1280/AB medium used for fold induction calculations was 8100 RLU.

It has also been observed that exogenous AI-2 signal detected by bioluminescence reporter strains like the wild-type *V. harveyi* BB170, which produces and detects its own AI-2, decreases between 5 and 5 ½ hours after inoculation (DeKeersmaecker and Vanderleyden, 2003; Ren *et al.*, 2001; Surette and Bassler, 1998). The reduction of exogenous AI-2 has been associated with interference from endogenous AI-2 of the reporter strain which contributes to < 1% of total bioluminescence signal detected (DeKeersmaecker and Vanderleyden, 2003). DeKeersmaecker and Vanderleyden (2003) therefore recommended that in investigating the effects of exogenous AI-2 on the fold induction of bioluminescence in *V. harveyi* over a time-period, AI-2 production must be assayed at selected time points. This will allow the observation of peak and decline in AI – 2 production and their effect on bioluminescence. In the work presented here, fold induction of bioluminescence in *V. harveyi* NCIMB 1280 by exogenous AI-2 donated by 2 DFU isolates, 028b *K. pneumoniae* and 005 *P. mirabilis* was monitored at selected time points (Figure 5.12A). It was observed that expression and detection of AI-2 peaked at time-point 3 (Figure 5.12AB). Addition of commercially prepared *N*-(3-hydroxybutanoyl) HSL to exogenous AI-2 to increase bioluminescence in *V. harveyi* NCIMB 1280 showed similar pattern in the luminescence of the biosensor reference strain without necessarily increasing fold induction. However, the values obtained for the AI-2 + HAI-1 assay in Figure 5.13B were inconsistent but generally lower than (except 110.5 for  $FI_{Kp}$  (AI-2+HAI-1) and 46.1 for  $FI_{Pm}$  (AI-2+HAI-1)) those obtained for AI-2 assay in Figure 5.13A



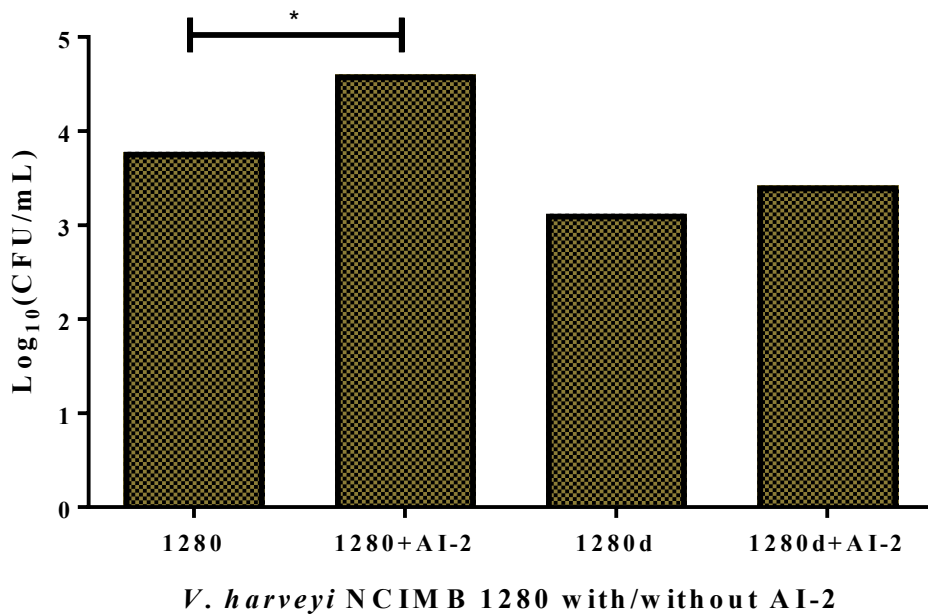
**Figure 5.12** Fold induction of luminescence of *V. harveyi* NCIMB 1280 reporter strain over a period of 24 hours; (A). Expression and detection of AI-2 only and; (B) combined effect of AI-2 and HAI-1 (*N*-(3-hydroxybutanoyl) HSL) on the induction of luminescence in biosensor reporter strain. Combined AI-2 and HAI-1 activity was monitored at 7 time-points using the GloMax<sup>®</sup>-Multi Detection system luminescence plate assay.

The difference between the fold induction of bioluminescence in the *V. harveyi* NCIMB 1280 biosensor reporter strain by AI-2 only and AI-2 + HAI-1 in Figure 5.13AB was statistically insignificant ( $p > 0.28$ ). The typical domed-shaped pattern of AI-2 induction of bioluminescence in *V. harveyi* reporter strains has been observed in previous works (Rickard *et al.*, 2010; Schneider *et al.*, 2002).

#### 5.4.4 Effect of exogenous AI-2 in biofilm formation by *V. harveyi* reference strains

The addition of exogenous AI-2 extracted from DFU isolates on *V. harveyi* in a biofilm co-culture was performed to assess the effect of quorum sensing on biofilm formation. Biofilm formation by *V. harveyi* strains NCIMB 1280 and NCIMB 1280<sub>d</sub> in the presence or absence of exogenous AI-2 was estimated using the biofilm growth check (BGC) step of the MBECTM assay as outlined in section 2.4.3.1. BGC was expressed as CFU/mL and a graph of Log<sub>10</sub> (CFU/mL) plotted both the wild-type and mutant *V. harveyi* strains (Figure 5.13). It was observed that, addition of exogenous AI-2 increased biofilm formation by 17.0% in the wild-type (NCIMB 1280) and 8.8% in the mutant (NCIMB 1280<sub>d</sub>) strain. Despite the addition of exogenous AI-2 to the NCIMB 1280<sub>d</sub> mutant strain, biofilm formation by the

wild-type strain was 9.5% more than that produced by the mutant strain in the presence of AI-2. The difference between biofilm biomass formed by the wild-type NCIMB 1280 in the presence of exogenous AI-2 (1280+AI-2) and that produced by the wild-type only (1280) was found to be statically significant by One Sample (two-tailed) *t* test ( $p = 0.0014$ ).

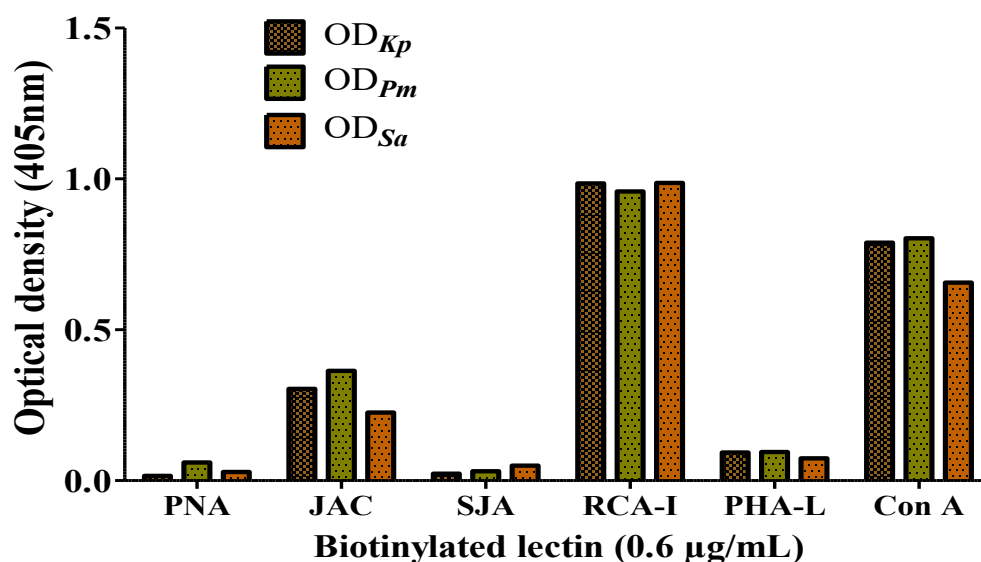


**Figure 5.13** Biofilm formation by *V. harveyi* NCIMB 1280 reference strain before and after mutagenesis and addition of exogenous AI-2. The wild-type *V. harveyi* NCIMB 1280 and dim mutant *V. harveyi* NCIMB 1280 strains have been denoted as 1280 and 1280d respectively. One Sample *t* test between 1280 and 1280+AI-2 was statistically significant ( $*p = 0.0014$ ).

## 5.5 Bacterial cell-cell interaction in biofilm formation

The expression and types of cell-surface polysaccharides during biofilm formation can provide important information in the characterisation of the biofilm for diagnostic and treatment purposes (Bales *et al.*, 2013; Tielker *et al.*, 2005; Winzer *et al.*, 2000). In the present study, an attempt was made to characterise the polysaccharides that are expressed by *K. pneumoniae* and *P. mirabilis* during biofilm formation using an ELISA-based technique called enzyme-linked lectinsorbent assay (ELLA). The presence of *K. pneumoniae* and *P. mirabilis* cell-surface polysaccharides which contain galactose and galacturonic acid monomers was determined by a panel of seven biotinylated lectins with specificity for those carbohydrates (Figure 5.14). From Figure 5.14, it can be seen that the lectin with high affinity

binding to corresponding glycans on the cells surfaces of *K. pneumoniae* and *P. mirabilis* biofilm extracts as well as the *S. aureus* (positive control) strain was *Ricinus communis Agglutinin I* (RCA-I) followed by *Concanavalin A* (CON A) and *Jacalin* (JAC). This implies that the exopolysaccharides, capsular polysaccharides and other protein-binding polysaccharides produced by *K. pneumoniae* and *P. mirabilis* DFU isolates contain glucose, mannose, N-acetyl D-galactosamine and galactose sugar monomers. *Phaseolus vulgaris Leucoagglutinin* (PHA-L) which binds to complex sugars also showed positive results which indicated the presence of sugars other than those detected by RCA-I, CON or JAC. In Table 5.2 it can be seen that, *Peanut Agglutinin* (PNA) could only detect galactose on the cell-membrane of *P. mirabilis* but not on *K. pneumoniae* or *S. aureus*. There was no detectable binding between *Sophora japonica* (SJA) and any of the bacterial cells.



**Figure 5.14** Specificity of carbohydrate-binding lectin to polysaccharides on whole cells of *K. pneumoniae* (OD<sub>Kp</sub>), *P. mirabilis* (OD<sub>Pm</sub>) and *S. aureus* (OD<sub>Sa</sub>). *S. aureus* was used as a positive control for Con A as demonstrated previously (Dharod, 2010).



**Table 5–2** Scoring\* system for whole cell glycan–lectin interactions using biotinylated lectins

Bacterial Strain	Biotinylated lectins					
	PNA	JAC	SJA	RCA	PHA–L	CON A
<i>K. pneumoniae</i>	–	+++	–	+++	+	+++
<i>P. mirabilis</i>	+	+++	–	+++	+	+++
<i>S. aureus</i>	–	+++	–	+++	+	+++

\*– A positive ELLA result was scored as (+) and a negative result as (–). Scoring of the assay was done using the following range as a guide as described by Dharod (2010): 0 – 0.05 represents (–); 0.06 – 0.14 represents (+); 0.15– 0.22 represents (++); and 0.23 and above represent (+++).

## 5.6 Effects of antimicrobial agents and quorum sensing inhibitors on biofilms

In the present study, the effect of 3 quorum sensing inhibitors (QSI); cinnamaldehyde (CIN), baicalin hydrate (BH) and (Z)–4–Bromo–5–(bromomethylene)–2(5H)–furanone (2(5H)–furanone) were assessed on their ability to inhibit *K. pneumoniae* and *P. mirabilis* biofilms. CIN, BH and 2(5H)–furanone are cyclic organic compounds which are derivatives of natural compounds that have widely been used as anti–quorum sensing agents (Brackman and Coenye, 2015; Brackman *et al.*, 2009; Christensen *et al.*, 2012; Romano *et al.*, 2013; Ren *et al.*, 2001). The MICs of baicalin hydrate, cinnamaldehyde and 2(5H)–furanone were determined in two–fold dilution assays using the MBEC™ assay as outlined in section 2.4.3.5. Sub–inhibitory concentrations of BH, CIN and 2(5H)–furanone selected for synergy assays were 2500 µg/mL, 1000 µM (132.06 µg/mL) and 250 µg/mL respectively. The combined effects of BH, CIN and 2(5H)–furanone (at sub–inhibitory concentrations) and ceftazidime and levofloxacin (at minimum inhibitory concentrations) were assessed on *K. pneumoniae* and *P. mirabilis* biofilms (Tables 5.3, 5.4 and 5.5). In two–fold dilution assays, the synergy between the antibiotics (ceftazidime and levofloxacin), and the QSIs (BH, CIN and 2(5H)–furanone), and the antimicrobial peptides (polymyxin B and polymyxin B nonapeptide) was also assessed (Tables 5.6 and 5.7).

### 5.6.1 Synergistic effects of anti-biofilm agents and antibiotics on DFU biofilms

The synergy between anti-biofilm agents such as baicalin hydrate, cinnamaldehyde, hamamelitannin and 2(5H)-furanone have been evaluated as alternate strategies to eradicate biofilms (Brackman *et al.*, 2011; Ghosh *et al.*, 2013; Janssens *et al.*, 2008). The current study evaluated the combinatorial effects of 3 QSIs, baicalin hydrate, cinnamaldehyde and 2(5H)-furanone and 2 antibiotics, ceftazidime and levofloxacin on 2 DFU biofilm-forming isolates, *K. pneumoniae* and *P. mirabilis*. In 2 separate assays, the synergy between 2 antimicrobial peptides (AMP); polymyxin B (PMB) and its derivative polymyxin B nonapeptide (PMBN) and CAZ/LEV in the inhibition of *K. pneumoniae* and *P. mirabilis* biofilms was also assessed.

**Table 5–3** The combined effect of BH\* and CAZ/LEV on *K. pneumoniae* and *P. mirabilis* biofilms

Bacterial strain	Antibiotic	Log <sub>10</sub> reduction		
		MIC (µg/mL)	MBC <sub>50</sub> (µg/mL) <sup>a</sup>	MBEC <sub>50</sub> (µg/mL) <sup>a</sup>
<i>K. pneumoniae</i>	Ceftazidime	640	2560	5120
	Levofloxacin	40	1280	2560
<i>P. mirabilis</i>	Ceftazidime	2560	5120	5120
	Levofloxacin	40	640	1280

\* – Sub-inhibitory of concentration of BH was maintained at 2500 µg/mL while concentrations of CAZ and LEV at 5120 µg/mL, were diluted in a two-fold dilution across the rows (from 1 to 10) of the 96-well base of the MBEC™ P&G plate. Columns 11 and 12 were used as positive controls for biofilm growth check.

<sup>a</sup> – Log<sub>10</sub> reduction assay at which ≥ 50% bacterial cells were killed or eradicated.

Table 5.3 shows the MIC values for CAZ and LEV in the presence of BH (2500 µg/mL) which resulted in complete inhibition of *K. pneumoniae* and *P. mirabilis* biofilms. Log<sub>10</sub> reduction assays also showed the MBC and MBEC at which 50% or more *K. pneumoniae* and *P. mirabilis* biofilm cells were prevented from growing on agar plates or eradicated by the combined activity of BH and CAZ/LEV. The MBC and MBEC values were 2 – 16 times more than their respective MICs.

The MIC values in Table 5.4 were the concentrations of CAZ and LEV in the presence of CIN (132.06 µg/mL) which resulted in complete inhibition of *K. pneumoniae* and *P. mirabilis* biofilms from forming on the pegs of the MBEC™ lid. However, the concentrations of CAZ and LEV needed to prevent ≥ 50% biofilm cells from growing on agar plate or eradicated were between 2 to 8 times more than their respective MICs.

**Table 5–4** The combined effect of CIN\* and CAZ/LEV on *K. pneumoniae* and *P. mirabilis* biofilms

Bacterial strain	Antibiotic	MIC (µg/mL)	Log <sub>10</sub> reduction	
			MBC <sub>50</sub> (µg/mL) <sup>a</sup>	MBEC <sub>50</sub> (µg/mL) <sup>a</sup>
<i>K. pneumoniae</i>	Ceftazidime	640	1280	2560
	Levofloxacin	40	640	2560
<i>P. mirabilis</i>	Ceftazidime	640	1280	5120
	Levofloxacin	20	640	2560

\* – Sub-inhibitory concentration of CIN was maintained at 1000 µM (132.06 µg/mL) while concentrations of CAZ and LEV at 5120 µg/mL, were diluted in a two-fold dilution across the rows (from 1 to 10) of the 96-well base of the MBEC™ P&G plate. Columns 11 and 12 were used as positive controls for biofilm growth check.

<sup>a</sup> – see footnote of Table 5.3 for details on Log<sub>10</sub> reduction.

**Table 5–5** The combined effect of 2(5H)–furanone\* and CAZ/LEV on *K. pneumoniae* and *P. mirabilis* biofilms

Bacterial strain	Antibiotic	Log <sub>10</sub> reduction		
		MIC (µg/mL)	MBC <sub>50</sub> (µg/mL) <sup>a</sup>	MBEC <sub>50</sub> (µg/mL) <sup>a</sup>
<i>K. pneumoniae</i>	Ceftazidime	10	40	40
	Levofloxacin	10	640	640
<i>P. mirabilis</i>	Ceftazidime	20	320	5120
	Levofloxacin	10	640	2560

\* – Concentration of 2(5H)–furanone was maintained at 500 µg/mL whereas concentrations of CAZ and LEV at 5120 µg/mL were diluted in two–fold across the rows (from 1 to 10) of the 96–well base of the MBEC™ P&G plate. Columns 11 and 12 were used as positive controls for biofilm growth check.

<sup>a</sup> – see footnote of Table 5.3 for details on Log<sub>10</sub> reduction.

In Table 5.5, the MBC and MBEC of CAZ and LEV in the presence of 2(5H)–furanone needed to prevent *K. pneumoniae* and *P. mirabilis* growth on agar or eradicate their biofilm were 4 – 256 times more than their individual MICs. The MICs of CAZ and LEV in the presence of 2(5H)–furanone were also found to be 2 – 128 times lower than those obtained when combined with BH or CIN.

Similarly, the MBECs of CAZ and LEV needed to eradicate *K. pneumoniae* and *P. mirabilis* biofilms by 50% or more in the presence of sub–inhibitory concentrations of PMB and PMB were 16 – 64 times higher than their individual MICs (Appendix B3 and 4).

### 5.6.1.1 Evaluation of synergy between QSI/AMP and antibiotics

In order to establish synergism between the QSI/AMP and the antibiotics in inhibiting *K. pneumoniae* and *P. mirabilis* biofilms, fractional inhibitory concentration (FIC) index for each synergy pair was calculated according to the formula described by Ruden *et al.*, (2009) as mentioned in Chapter 2, section 2.6.3. Tables 5.6 and 5.7 show the summary of FIC indices determined for each synergy pair.

**Table 5–6** Determination of the effects of antimicrobial combinations on biofilm–forming DFU isolates

Bacterial Strain	QSI/AMP	MIC pair <sup>a</sup>		Synergy pair <sup>a</sup>		FIC <sub>CAZ</sub> Index <sup>†</sup>
		QSI/AMP (µg/mL)	CAZ (µg/mL)	QSI/AMP (µg/mL)	CAZ (µg/mL)	
<i>K. pneumoniae</i>	BH	12500	5120	2500	640	0.325
	CIN	660.8	5120	132.06	640	0.325
	2(5H)–furanone	500	5120	250	10	0.593
	PMB	500	5120	100	40	0.21
	PMBN	500	5120	100	40	0.21
<i>P. mirabilis</i>	BH	12500	640	2500	640	1.2
	CIN	660.8	640	132.06	640	1.2
	2(5H)–furanone	500	640	250	20	0.813
	PMB	500	640	100	10	0.7
	PMBN	500	640	100	10	0.7

<sup>†</sup> – FIC<sub>CAZ</sub> index determined for each combination assay between CAZ and sub–inhibitory concentrations of QSI/AMP. Interpretation of FIC index: Synergy was defined as  $\leq 0.5$ ; FIC index  $> 0.5 < 2.0$  was indicative of “additive effect”, and FIC index above 2.0 was indicative of “antagonistic effect.”

<sup>a</sup> – MIC pair: individually determined MICs for each QSI/AMP and antibiotic (CAZ). Synergy pair: MIC of each QSI/AMP and antibiotic (CAZ) determined in a synergy assay.

**Table 5–7** Effects of antimicrobial combinations on biofilm–forming DFU isolates

Bacterial Strain	QSI/AMP	MIC pair <sup>a</sup>		Synergy pair <sup>a</sup>		FIC <sub>LEV</sub> index <sup>†</sup>
		QSI/AMP (µg/mL)	LEV (µg/mL)	QSI/AMP (µg/mL)	LEV (µg/mL)	
<i>K. pneumoniae</i>	BH	12500	40	2500	40	1.2
	CIN	660.8	40	132.06	40	1.2
	2(5H)–furanone	500	40	250	10	0.75
	PMB	500	40	100	40	1.2
	PMBN	500	40	100	40	1.2
<i>P. mirabilis</i>	BH	12500	40	2500	40	1.2
	CIN	660.8	40	132.06	20	0.7
	2(5H)–furanone	500	40	250	10	0.75
	PMB	500	40	100	10	1.2
	PMBN	500	40	100	10	1.2

<sup>†</sup> – FIC index determined for synergy between LEV and sub–inhibitory concentrations of QSI/AMP. Refer to footnote \* for interpretation of FIC index reference values.

<sup>a</sup> – MIC pair: individually determined MICs for each QSI/AMP and antibiotic (LEV). Synergy pair: MIC of each QSI/AMP and antibiotic (LEV) determined in a synergy assay.

FIC index values obtained for the antimicrobial combination assays between CAZ/LEV and all quorum sensing inhibitors, BH, CIN, 2(5H)–furanone and antimicrobial peptides (PMB and PMBN) showed synergistic or additive effects (Tables 5.6 and 5.7). For the purpose of this study, the definitions of synergism, additive and antagonistic effects of antimicrobial combinations adapted from the definitions provided by Thellin *et al.* (2015). Synergy or synergistic effect was indicative when the effects of an antibiotic and an antimicrobial agent in a combination assay were greater than the sum of their individual effects. Additive effect on the other hand was indicative when the combined effect of an antibiotic and an antimicrobial agent in a combination assay was not greater than the sum of their individual effects but still greater than the effect of each individual antimicrobial agent. Finally, an

antagonistic effect was indicated when the effects of an antibiotic and an antimicrobial agent in a combination assay were lower than their individual effects (Bassolé and Juliani, 2012).

In the current study, it was observed that the antibacterial effects of the synergy pair in some combination assays resulted in the reductions of the MIC values of antibiotics compared to when they were individually used. For example, in the case of CAZ, the MIC in the combination assays against *K. pneumoniae* biofilms was 8 – 512 times lower than when it was used alone (Table 5.6). When tested against *P. mirabilis*, the MIC of CAZ in combination assays involving 2(5H)–furanone, PMB and PMBN, was 32 – 64 times lower than when used alone (Table 5.6). The MIC of CAZ in combination assays with BH and CIN against *P. mirabilis* biofilms remained unchanged (640 µg/mL). With the exception of the combination assay with 2(5H)–furanone against *K. pneumoniae* (which was near–synergy), CAZ combinations assays with BH, CIN, PMB and PMBN resulted in synergy effects with FIC indices  $\leq 0.5$  (Table 5.6). On the other hand, CAZ combination assays with BH, CIN, 2(5H)–furanone, PMB and PMB resulted in additive effects.

When tested against *K. pneumoniae*, the MIC of LEV in the presence of 2(5H)–furanone was 4 times lower than when used on its own (Table 5.7). However, the MIC of LEV remained unchanged (the same as when used alone) when tested against *K. pneumoniae* in combinations with BH, CIN, PMB and PMBN. When tested against *P. mirabilis* however, the MIC of LEV was 1 – 4 times lower than when used alone (Table 5.7). All the combination assays involving LEV resulted in additive effects with the exception of combinations with 2(5H)–furanone against *K. pneumoniae* and *P. mirabilis* respectively which resulted in near–synergy effect.

### **5.6.2 Antimicrobial effects of wound dressings on diabetic foot isolates**

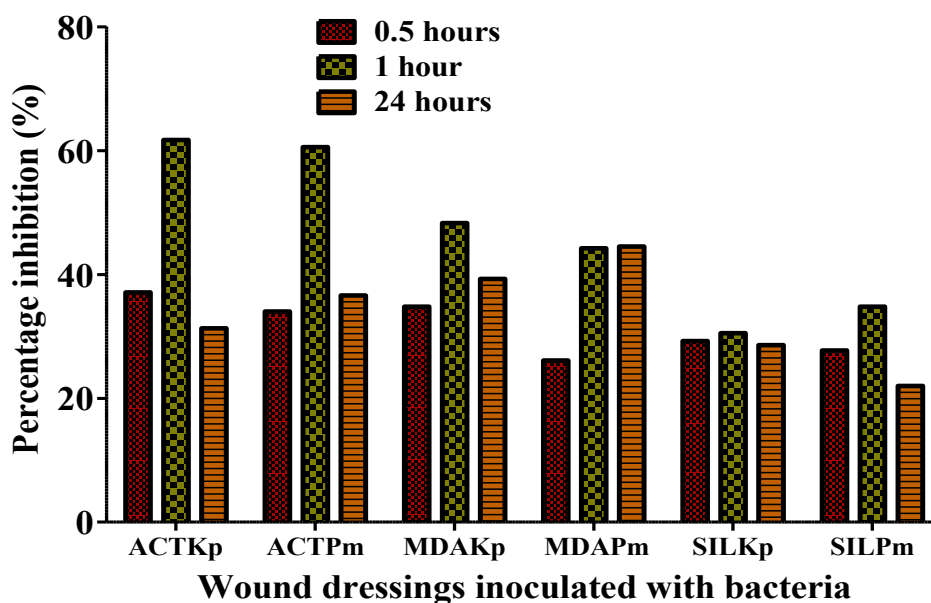
The antibacterial effects of 3 wound dressings namely; Acticoat® (ACT), Silvercel® (SIL, silver–impregnated), and Medihoney™ Apinate (MDA, honey–impregnated) were assessed on 2 biofilm–forming DFU isolates, *K. pneumoniae* and *P. mirabilis*. In order to compare their antibacterial effects, a 4th wound dressing called Atrauman (ATR), with no antibacterial activity was used as a growth control check. The inhibition of *K. pneumoniae* and *P. mirabilis* biofilm formation was evaluated by a 6–well plate assay and a standard agar

method. In both assays, the antimicrobial effects of individual wound dressings and in combination with antibiotics (CAZ and LEV) were assessed.

#### 5.6.2.1 6–well plate assay

In Figure 5.15, the inhibitory effects of ACT, MDA and SIL at 3 selected time points (30 minutes, 1 hour and 24 hours after incubation) were compared to that of ATR which showed no inhibition at any of the selected time points. The selection of the 3 time points was based on manufacturers' recommendations. The antimicrobial efficacy of ACT has been suggested to attain peak levels within 1 hour of application (Keene, 2002; Smith & Nephew Data on file report 0810018; Smith & Nephew report reference DS/08/062/R2; Wright *et al.*, 1998). On the other hand, MDA has been found to have sustained antimicrobial effect up to 7 days following initial application (Bateman and Graham, 2007; George and Cutting, 2007; Gethin and Cowan, 2005). The non–adherent SIL has also been found to sustain its efficacy up to 7 days after initial application (Clark and Bradbury, 2010). Interestingly, all 3 wound dressings were most effective 1-hour post–incubation with *K. pneumoniae* and *P. mirabilis*. ACT was the most effective by inhibiting *K. pneumoniae* and *P. mirabilis* biofilm formation by 60% or more at 1-hour post–incubation. Percentage inhibition by Acticoat<sup>®</sup> against *K. pneumoniae* (ACT<sub>Kp</sub>) and Acticoat<sup>®</sup> against *P. mirabilis* (ACT<sub>Pm</sub>) at 1 hour was found to be statistically significant ( $p = 0.0105$ ) compared to that of SIL and MDA. Although inhibition of biofilm after 24 hours was inconsistent among the 3 wound dressings, Medihoney<sup>™</sup> Apinate against *K. pneumoniae* (MDA<sub>Kp</sub>) and Medihoney<sup>™</sup> Apinate against *P. mirabilis* (MDA<sub>Pm</sub>) showed sustained antimicrobial effect. However, the sustained antimicrobial effect of MDA was inadequate to significantly or completely inhibit *K. pneumoniae* and *P. mirabilis* biofilms after a 24–hour incubation period ( $p = 0.0921$ ).

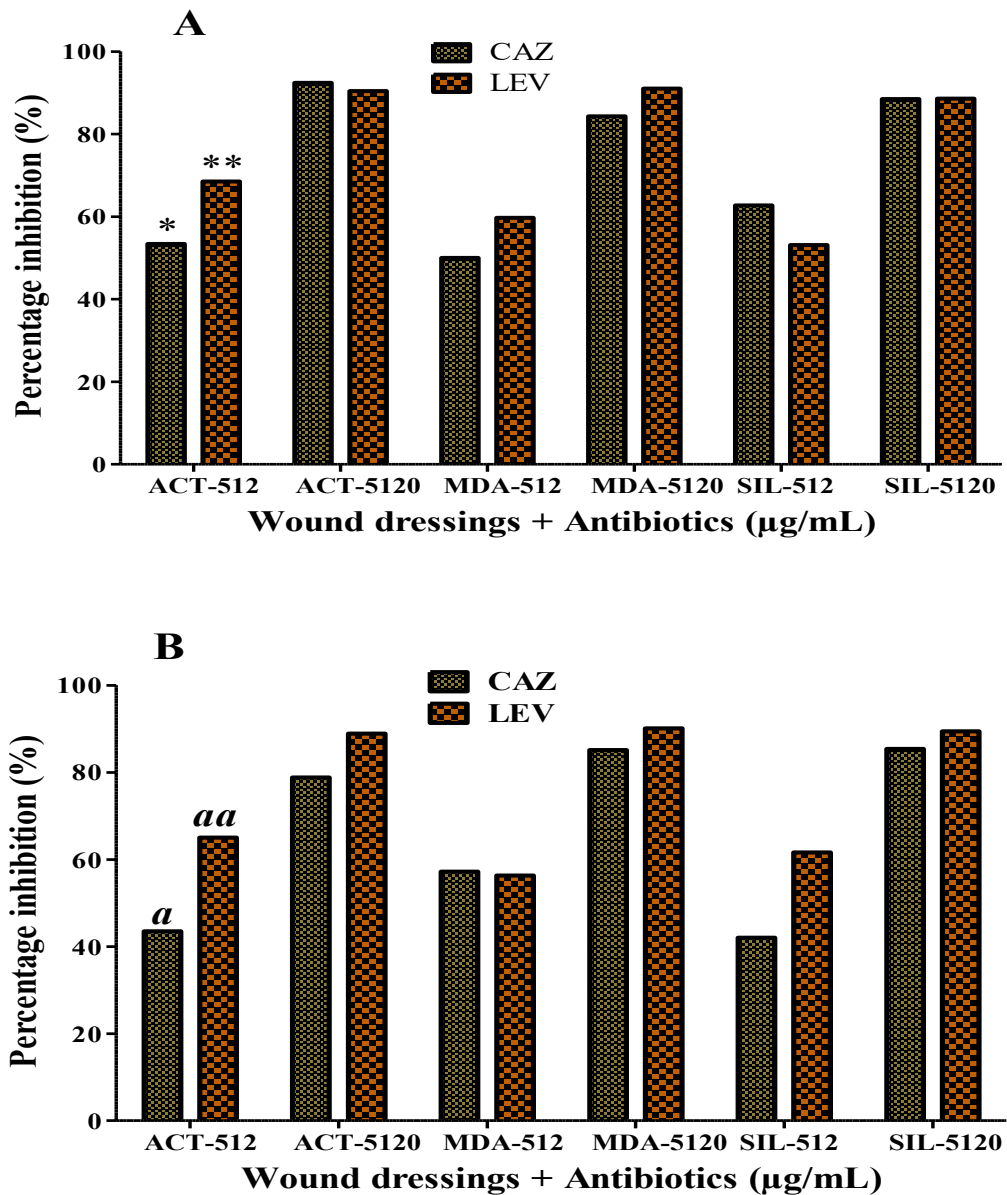




**Figure 5.15** Effect of wound dressings on biofilm formation by *K. pneumoniae* and *P. mirabilis*. Percentage inhibition by SIL<sub>Kp</sub> and SIL<sub>Pm</sub> was statistically significant ( $p = 0.0071$ , two-way ANOVA).

In Figure 5.16, the combined effect of wound dressings and the two antibiotics, CAZ and LEV at 512 and 5120  $\mu\text{g/mL}$  were evaluated in the inhibition of *K. pneumoniae* and *P. mirabilis* biofilms after a period of 24 hours of incubation. It was observed that the combined effect of the 3 wound dressings, ACT, MDA and SIL in the presence of 5120  $\mu\text{g/mL}$  of CAZ or LEV completely inhibited *K. pneumoniae* and *P. mirabilis* biofilms. In the case of Acticoat® against *P. mirabilis* (ACT<sub>Pm</sub>), which recorded the least inhibition, 78.8%, for CAZ among the 3 wound dressing – antibiotic combination assays, a further test on agar to detect bacterial growth was negative. It was also observed that, the combination of ACT<sub>Kp</sub> and LEV<sub>512</sub>, and SIL<sub>Kp</sub> and CAZ<sub>512</sub> inhibited *K. pneumoniae* biofilms by more than 60% (Figure 5.16A). Similarly, the combined effect of ACT<sub>Pm</sub> and LEV<sub>512</sub>, and SIL<sub>Pm</sub> and LEV<sub>512</sub> inhibited *P. mirabilis* biofilm by more than 60% (Figure 5.16B). The combined effect of LEV<sub>512</sub> and ACT, MDA or SIL (in Figure 5.16A) in the inhibition of *K. pneumoniae* biofilms was found to be statistically significant ( $p = 0.0049$ ) while that of CAZ<sub>512</sub> and ACT, MDA was not ( $p = 0.4013$ ) when compared with the individual effects of ACT, MDA and SIL (in Figure 5.15) 24-hour post-incubation. On the other hand, the combined effects of CAZ<sub>512</sub> and ACT, MDA or SIL, and that of LEV<sub>512</sub> and ACT, MDA or SIL (in Figure 5.16B) were also found to be statistically significant ( $p = 0.0432$  and  $p = 0.0046$  respectively) when compared

with the individual effects of ACT, MDA and SIL (in Figure 5.15) in the inhibition of *P. mirabilis* biofilms 24 hours post-incubation.



**Figure 5.16** Combined effects of wound dressings and antibiotics in inhibiting; (A). *K. pneumoniae* biofilm, \* $p = 0.4013$  and \*\* $p = 0.0049$  represent combined effects of CAZ<sub>512</sub> and ACT, MDA and SIL, and LEV<sub>512</sub> and ACT, MDA or SIL respectively in the inhibition of *K. pneumoniae* biofilms. (B). *P. mirabilis* biofilm, <sup>a</sup> $p = 0.0432$  and <sup>aa</sup> $p = 0.0046$  represent combined effects of CAZ<sub>512</sub> and ACT, MDA and SIL, and LEV<sub>512</sub> and ACT, MDA or SIL respectively in the inhibition of *P. mirabilis* biofilms.

In order to determine synergism between the combined inhibitory effects of the 3 wound dressings used in this study, Acticoat®, Medihoney™ Apinate and Silvercel®, and the 2 antibiotics, ceftazidime and levofloxacin, the FIC index for each synergy assay was calculated as described earlier (Chapter 2, section 2.6.3). Details of each combination assay can be found in Tables 5.8 and 5.9

**Table 5–8** Antimicrobial effect of combinations wound dressings and antibiotics on biofilm-forming DFU isolates

Bacterial Strain	Wound dressings (WD)	Individual pair <sup>a</sup>		Synergy pair <sup>a</sup>		FIC <sub>CAZ</sub> index <sup>1</sup>
		WD PI (%)	CAZ PI (%)	WD PI (%)	CAZ PI (%)	
<i>K. pneumoniae</i>	ACT–512	48.6	70	53.4	55	1.66
	ACT–5120	48.6	70	92.4	70	2.01
	MDA–512	58.0	70	50.0	55	1.74
	MDA–5120	58.0	70	84.3	70	2.03
	SIL–512	57.2	70	62.7	55	1.96
	SIL–5120	57.2	70	88.5	70	2.08
<i>P. mirabilis</i>	ACT–512	43.1	65	43.5	65	1.33
	ACT–5120	43.1	65	78.8	70	1.79
	MDA–512–	47.0	65	57.2	65	1.6
	MDA–5120	47.0	65	85.1	70	1.94
	SIL–512	49.3	65	42.0	65	1.41
	SIL–5120	49.3	65	85.4	70	1.98

<sup>1</sup> – FIC index determined for the interpretation of synergism between CAZ and wound dressings. Interpretation of FIC index: Synergy was defined as  $\leq 0.5$ ; FIC index  $> 0.5 < 2.0$  was indicative of “additive effect”, and FIC index above 2.0 was indicative of “antagonistic effect.”

<sup>a</sup> – Individual pair: individually determined MICs and inhibitory concentrations for the antibiotic (CAZ at 5120  $\mu\text{g}/\text{mL}$  for *K. pneumoniae* and 640  $\mu\text{g}/\text{mL}$  for *P. mirabilis*) and each wound dressing respectively. Synergy pair: pre-determined concentrations of the antibiotic

(CAZ at 512 and 5120  $\mu\text{g/mL}$ ) and the MIC of each wound dressing in a synergy assay. All MICs and inhibitory concentrations were expressed as percentage inhibitions. In Table 5.8, the FIC index values obtained for combination assays between the wound dressings and CAZ at 5120  $\mu\text{g/mL}$  (70% inhibition) resulted in antagonistic effects against *K. pneumoniae* but not *P. mirabilis*. This implies that, the effect of the concentration of CAZ at 5120  $\mu\text{g/mL}$  was not complementary to that of the wound dressings. However, a different scenario was observed in the combination assays involving LEV and the wound dressings in Table 5.9. All combination assays resulted in additive effects suggesting LEV as antibiotic of choice in a combination therapy with wound dressings against DFU isolates.

**Table 5–9** Antimicrobial effect of combinations of wound dressings and antibiotics on biofilm–forming DFU isolates

Bacterial Strain	Wound dressings (WD)	Individual pair <sup>a</sup>		Synergy pair <sup>a</sup>		FIC <sub>LEV</sub> index <sup>†</sup>
		WD PI (%)	LEV PI (%)	WD PI (%)	LEV PI (%)	
<i>K. pneumoniae</i>	ACT–512	48.6	75	68.5	75	1.56
	ACT–5120	48.6	75	90.4	80	1.78
	MDA–512	58.0	75	59.7	75	1.57
	MDA–5120	58.0	75	91.0	80	1.91
	SIL–512	57.2	75	53.1	75	1.47
	SIL–5120	57.2	75	88.6	80	1.87
<i>P. mirabilis</i>	ACT–512	43.1	71	65.0	65	1.61
	ACT–5120	43.1	71	88.9	83.2	1.68
	MDA–512	47.0	71	56.3	65	1.53
	MDA–5120	47.0	71	90.1	83.2	1.74
	SIL–512	49.3	71	61.6	65	1.64
	SIL–5120	49.3	71	89.4	83.2	1.76

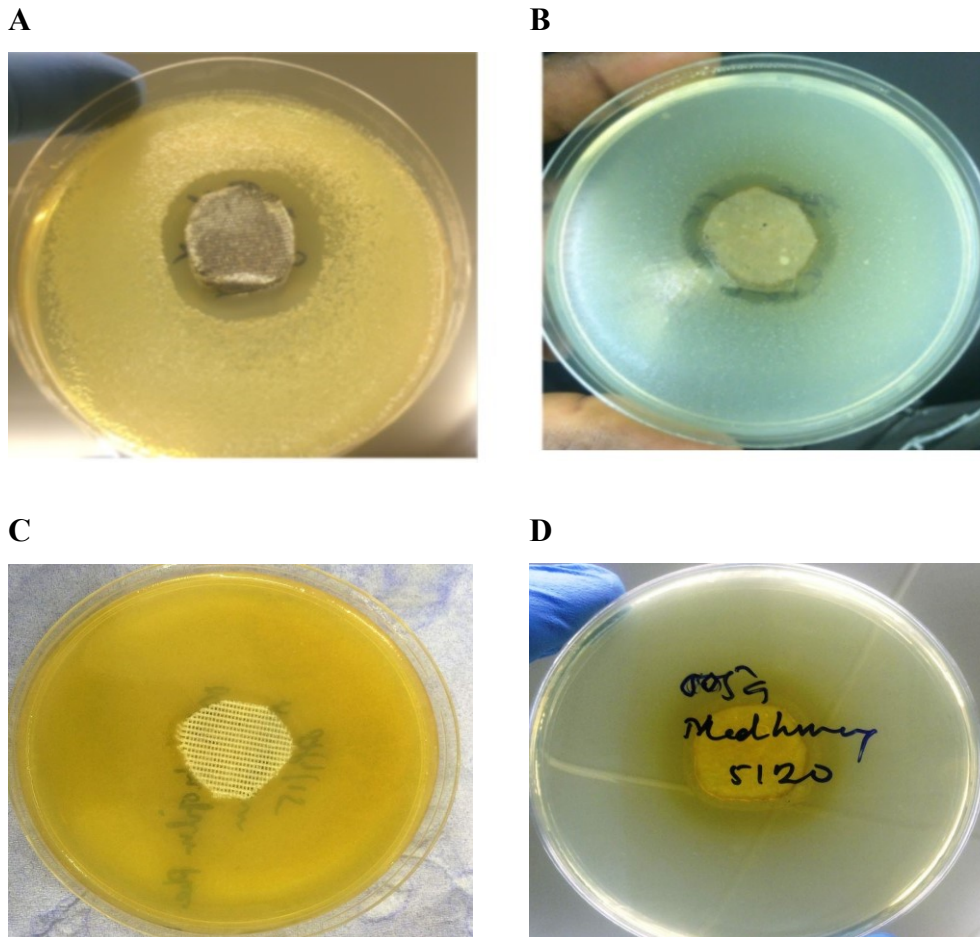
<sup>†</sup> – FIC index determined for the interpretation of synergism between LEV and wound dressings. Interpretation of FIC index: Synergy was defined as  $\leq 0.5$ ; FIC index  $> 0.5 < 2.0$  was indicative of “additive effect”, and FIC index above 2.0 was indicative of “antagonistic effect.”

<sup>a</sup> – Individual pair: individually determined MICs and inhibitory concentrations for the antibiotic (LEV at 640  $\mu\text{g}/\text{mL}$  for *K. pneumoniae* and 1280  $\mu\text{g}/\text{mL}$  for *P. mirabilis*) and each wound dressing respectively. Synergy pair: pre–determined concentrations of the antibiotic (LEV at 512 and 5120  $\mu\text{g}/\text{mL}$ ) and the MIC of each wound dressing in a synergy assay. All MICs and inhibitory concentrations were expressed as percentage inhibitions.

### 5.6.2.2 Standard agar assay

In the standard agar assay, the individual and combined effects of ACT, MDA and SIL and with CAZ and LEV were studied on *K. pneumoniae* and *P. mirabilis* quasi–biofilms on Mueller–Hinton agar (MHA) and Kolliphor® P 407 (KP 407) gel, a derivative of poloxamer

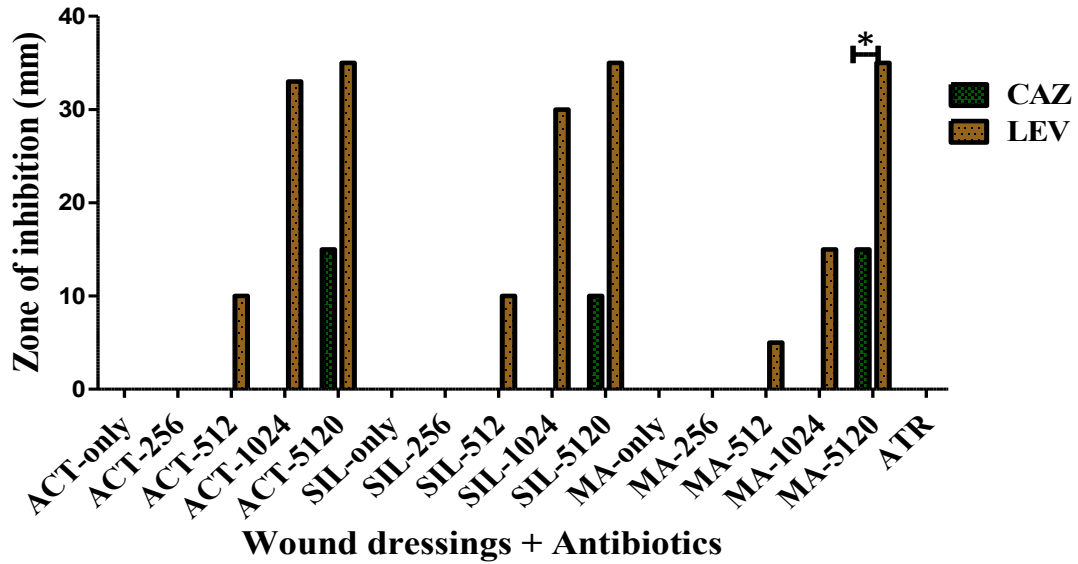
hydrogel. Unlike the 6-well plate assay in Figure 5.16, the individual effects of ACT, MDA and SIL showed no zone of inhibition (ZOI) on either MHA or KP 407 gels. Bacterial growth on KP 407 gels were less susceptible to ACT, MDA and SIL with smaller ZOIs than those grown on MHA. This is because poloxamer gels support the growth of biofilm than planktonic growth.



**Figure 5.17** Biofilm phenotypes of *P. mirabilis* in the presence of: (A) ACT with 1024  $\mu\text{g}/\text{mL}$  of LEV on MHA; (B) Medihoney with 512  $\mu\text{g}/\text{mL}$  of LEV on Kolliphor® P 407 gel (C) Atrauman showing no ZOI after 24 hours of growth on MHA; (D) Medihoney with 5120  $\mu\text{g}/\text{mL}$  of LEV on Kolliphor® P 407 gel;

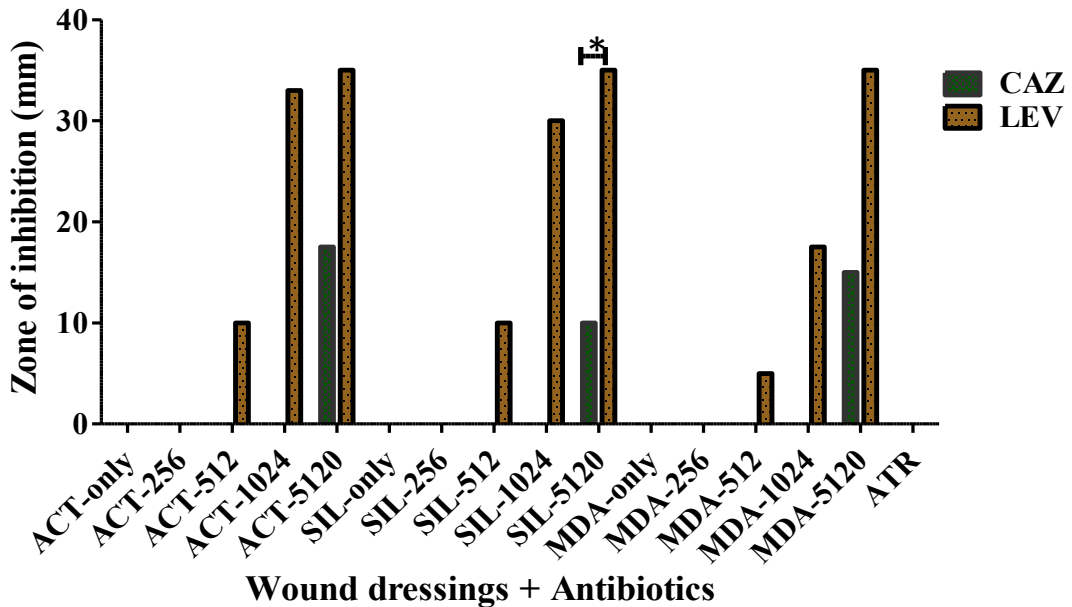
**A**

**Inhibition of *K. pneumoniae* biofilms on MHA**



**B**

**Inhibition of *P. mirabilis* biofilms on MHA**



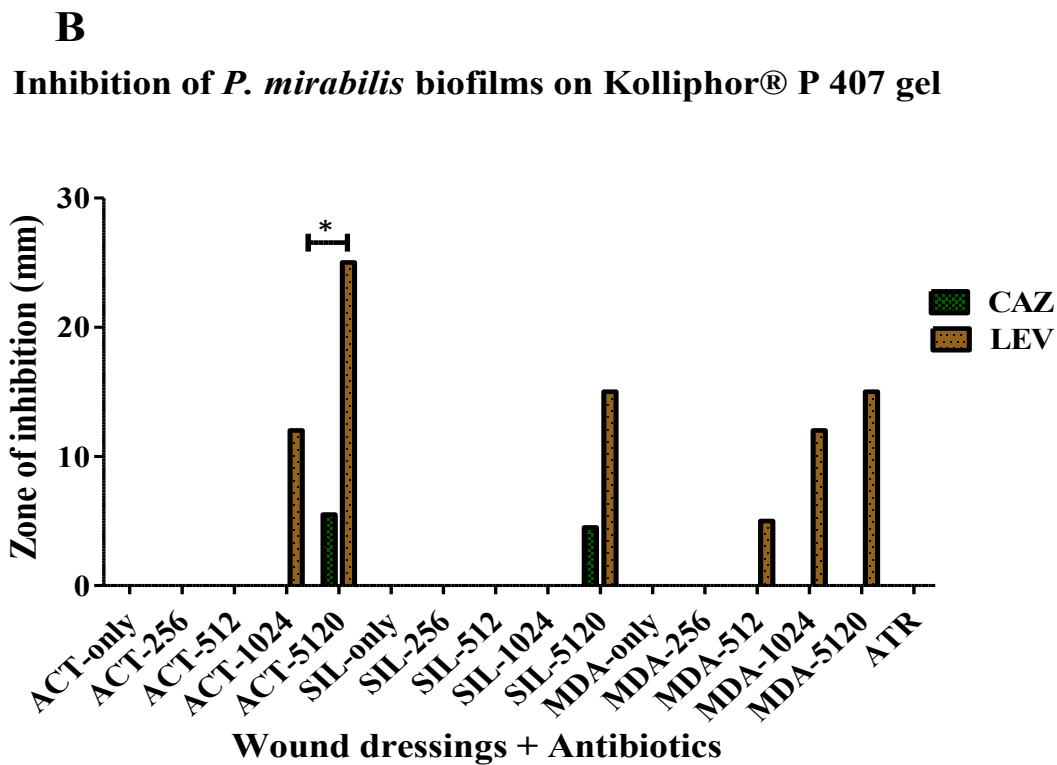
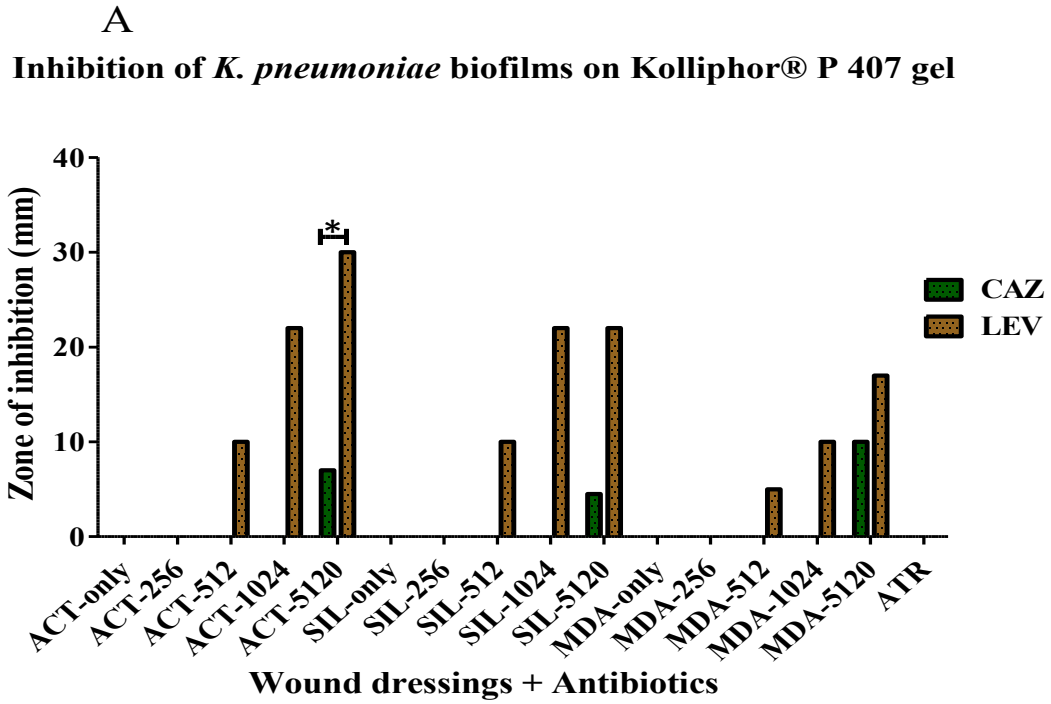
**Figure 5.18** Combined effects of wound dressings and antibiotics on (A) *K. pneumoniae* and (B) *P. mirabilis* biofilm. *K. pneumoniae* and *P. mirabilis* biofilm formation on MHA was significantly inhibited by LEV at 5120  $\mu\text{g/mL}$  ( $*p < 0.05$ ).

The combined effects of CAZ/LEV and wound dressings that showed significant ZOI on MHA and KP 407 gels were measured and compared (Figures 5.18 and 5.19). Wound dressings in the presence of CAZ and LEV from 512 to 5120 µg/mL showed significant ( $p = 0.0329$  and  $p = 0.0029$  respectively) inhibitory effect against *K. pneumoniae* and *P. mirabilis* with ZOIs between 5.0 and 35 mm on MHA (Figure 5.18A and B). Similarly, significant ( $p = 0.0153$  and  $p = 0.0028$ ) ZOIs on KP 407 gels against *K. pneumoniae* and *P. mirabilis* quasi-biofilms were between 4.5 and 30 mm at the concentrations of CAZ and LEV mentioned earlier (Figure 5.19A and B).

It was also observed that *K. pneumoniae* and *P. mirabilis* on MHA or KP 407 gels were more susceptible to LEV than CAZ (Figures 5.18 and 5.19). *K. pneumoniae* and *P. mirabilis* biofilm forming cells were found to be susceptible to three concentrations of LEV (512, 1024 and 5120 µg/mL) and only one concentration of CAZ (5120 µg/mL) in the combination assays. A similar scenario was observed in the 6-well plate assay (Figure 5. 16) except that *K. pneumoniae* and *P. mirabilis* were susceptible to CAZ (512 µg/mL) in all combination assays. In Figure 5.19B, none of the CAZ-MDA combination assays was effective against *P. mirabilis*.

It was not possible to calculate the FIC index for the combination assay using the standard agar technique as *K. pneumoniae* and *P. mirabilis* were completely resistant to all 3 wound dressings hence showed no ZOI. However, a reference range for interpretation of ZOI was deduced from the results of a similar work by Percival *et al.* (2007). In their work, Percival *et al.* (2007) compared the ZOIs of known antibiotic susceptible and resistant reference NCIMB strains which included *E. coli* NCIMB 8545, *P. aeruginosa* NCIMB 8626 and *P. aeruginosa* NCIMB 8506 respectively with clinical isolates. The range of ZOI associated with the antibiotic susceptible reference strains was between 1.9 and 17.5 mm. By this definition, all combination assays that resulted in ZOIs  $> 1.9$  mm were synergistic and those  $< 1.9$  were antagonistic. Further discussion on Chapter 5 can be found in the general discussion in Chapter 6.





**Figure 5.19** Combined effects of wound dressings and antibiotics on; (A) *K. pneumoniae* and (B) *P. mirabilis* biofilms. *K. pneumoniae* and *P. mirabilis* biofilm formation on Kolliphor® P 407 gel was significantly inhibited by LEV at 5120 µg/mL (\* $p < 0.05$ ).

## 5.7 Discussion

The evidence that the existence of cell-to-cell signalling processes that allow bacteria to regulate gene expression by producing, secreting, detecting and responding to extracellular signalling molecules has increased knowledge and enhanced the use of molecular techniques in the study cellular and molecular processes such as virulence gene expression, swarming and motility, biofilm formation, antibiotic resistance, horizontal gene transfer and evasion of host defence mechanisms (Antonova and Hammer, 2011; Bassler, 1999; Davies *et al.*, 1998; De Kievit *et al.*, 2000; De Kievit *et al.*, 2001; Hammer and Bassler, 2003; Tseng *et al.*, 2016; Waters and Bassler, 2005). For examples Davies *et al.* (1998) demonstrated that quorum sensing was required for *P. aeruginosa* cells to differentiate into complex structures called biofilms and mutation in the wild-type *P. aeruginosa* that resulted in *lasI* mutant strains could not differentiate resulting in abnormal biofilm production. In another study, Daniels *et al.* (2004) demonstrated that *swrI/swrR* quorum sensing system was responsible for the control of at least 28 genes in *Serratia liquefaciens* that leads to the production of serrawettin, a lipodepsipeptide biosurfactant responsible for producing swarming colonies. Induction of mutation in wild-type *S. liquefaciens* resulted in the loss of swarming ability which was restored after the exogenous addition of AHLs.

The idea that AI-2 – mediated QS is widely distributed among bacteria and hence involved in interspecies communication, have led to the discovery that, the biosynthetic pathway leading to the production of the precursors of AI-2 in *V. harveyi* are identical in *E. coli*, *S. enterica* serovar *typhimurium*, *V. harveyi*, *V. cholerae*, *Enterococcus faecalis*, *Neisseria meningitidis*, *Porphyromonas gingivalis*, and even in Gram-positive bacteria such as *S. aureus* (Schauder *et al.*, 2001; Winzer *et al.*, 2002; Xavier and Bassler, 2005b). In the current work presented here, BioCyc biosynthetic pathway tools (as outlined in section 2.8.6) revealed that, the pathway leading to AI-2 biosynthesis has already been found in *K. pneumoniae* and *P. mirabilis* and is identical to that of *V. harveyi* except in the final product (Bassler *et al.*, 1994; Chen *et al.*, 2002; Miller *et al.*, 2004). Upon PCR, the *luxS* synthase gene was found to be expressed by both *K. pneumoniae* and *P. mirabilis*. In order to determine the relatedness of the *luxS* genes expressed by *K. pneumoniae*, *P. mirabilis* and other speciated strains in the databases, partial DNA sequences of *K. pneumoniae* and *P. mirabilis* were used to perform a multiple sequence alignment and construct a cladogram (Figures 5.6 and C3 (in Appendix C).

The multiple sequence alignments in Figures C3, C4, C5 and C6 in Appendix C, showed some differences (in base pairs) in the variable and conserved regions of the 5' *luxS*, *lsrB* and *bssS/bssR* genes.

Differences (in DNA base pairs) in the variable regions of the above-mentioned quorum sensing regulated genes suggest that they are highly conserved in Gram-negative bacteria (Inoue and Takikawa, 2006). Highly conserved genes are useful in the grouping and typing of bacterial species and strains (Inoue and Takikawa, 2006). Similarly, differences in variable regions of the same gene may also indicate diverse functional roles in different bacterial species (Birkenmeier *et al.*, 1993). For examples, the variable regions of the *luxS* gene in *K. pneumoniae* may direct the transcription of quorum sensing genes for the synthesis of cell surface proteins for bacterial cell-cell attachment during biofilm formation and that of *P. mirabilis* may lead to the expression of genes that support nitrogen fixation. It can therefore be suggested that DNA sequence variations in the variable regions of the *luxS* gene confirm their specificity in binding to their respective receptor proteins during quorum sensing (Xavier and Bassler, 2005b). For example, *V. harveyi* AI-2 specifically binds to the periplasmic protein LuxP during QS regulation in *V. harveyi* while AI-2 produced by *S. enterica* serovar *typhimurium* binds to the ABC transporter protein, LsrB which internalises AI-2 in the cytoplasm for QS signal transduction (Xavier *et al.*, 2007).

In this study, the effect of exogenously produced quorum sensing molecules, AHL and AI-2, in the regulation of cellular activities in other bacteria was investigated. In the cross-feeding assay (as outlined in section 2.6.1.2), the biosensor reporter strain, *A. tumefaciens* NCIMB 14543 could detect exogenous acylated homoserine lactone produced by DFU isolates which resulted in blue pigmentation of *A. tumefaciens* NCIMB 14543 colonies on agar (Figure 5.10 A and B). In another assay (i.e., the cross-stimulation assay, outlined in section 2.6.1.4), AI-2 produced by DFU isolates, *K. pneumoniae* and *P. mirabilis* were detected by *V. harveyi* NCIMB 1280 reference strain which resulted in bioluminescence as a result of the activation of the *luxCDABE* operon (Bassler *et al.*, 1993). Bioluminescence in *V. harveyi* is QS-regulated as mentioned in section 5.3.2. The production, secretion, detection and response to QS molecules, AHL and AI-2 in bacteria confirm that quorum sensing as one of the mechanisms that regulate cellular activities in bacteria such as bioluminescence and biofilm formation (Figures 5.11 and 5.12).

The current study also investigated bacterial cell–cell interactions during *in vitro* biofilm formation using a panel of biotinylated lectins to identify corresponding protein–binding polysaccharides on the surfaces bacteria involved in biofilm formation. The results obtained for the glycan–lectin assay suggest that the detected protein–binding carbohydrates on the cell–surfaces of *K. pneumoniae* and *P. mirabilis* can be expressed *in vivo* during colonisation and biofilm formation in diabetic foot ulcers.

Attempts were made to develop antimicrobial formulations that effectively combine the efficacies of antibiotics such as ceftazidime and levofloxacin; antimicrobials such as polymyxin B and polymyxin B nonapeptide; and quorum sensing inhibitors such as baicalin hydrate, cinnamaldehyde and 2(5H)–furanone (Tables 5.6 and 5.7). In other assays, the combined effects of wound dressings and antibiotics were also assessed on diabetic foot isolates, *K. pneumoniae* and *P. mirabilis*, in inhibiting their growth either as planktonic cells or biofilm phenotypes (Figures 5.16 and 5.18). The combination of antibiotics and the other antibiofilm agents in the combination assays outlined in section 2.6.3 generated fractional inhibitory concentration indices (defined in section 2.6.3) that have been reported as benchmarks for the development of antimicrobial formulations that may provide alternative treatment for infections which otherwise cannot be treated using a single antimicrobial regimen (Ghosh *et al.*, 2013; Ruden *et al.*, 2009). This study therefore proposes that development of further combination assays based on the FIC indices obtained, using antibiotics such as ceftazidime and levofloxacin in combinations with other anti–quorum sensing and antibiofilm agents may provide alternate treatment with acceptable therapeutic concentrations that can significantly inhibit quorum sensing and biofilm formation in wounds.

# Chapter 6

## General Discussion, Limitations, Future Work and Conclusion

## 6.1 General Discussion

Diabetes mellitus (DM) is one of the major global health concerns which results in severe life-changing complications such as neuropathy, nephropathy, cardiovascular and peripheral vascular diseases and diabetic foot ulcers (International Diabetes Federation, 2015). It has been predicted that current global prevalence of 1 in 11 adults with the disease will change to 1 in 10 by the year 2040 with 75% of the disease among people living in developing countries (King *et al.*, 1998; International Diabetes Federation, 2015; Zimmet *et al.*, 2001). Lack of understanding and awareness of the disease have been identified as the major barriers to the effective prevention and management of the disease (International Diabetes Federation, 2015). A complex combination of genetic and environmental factors in addition to social deprivation makes diabetes more common in people of African, African-Caribbean and South Asian lineages (Chen *et al.*, 2012). Due to social deprivation and economic constraints, the major burden of DM and its complications are borne by patients living in developing countries (Abbott *et al.*, 2005; Chen *et al.*, 2012; International Diabetes Federation, 2015).

The work presented here studied diabetic foot ulcers (DFU) among diabetic patients who attended the Diabetes Centre, Komfo Anokye Teaching Hospital, KATH, Kumasi, Ghana from January 2001 to December 2004. It is also a follow-up on two previous cohort studies conducted by Jauhangeer (2006) and Dharod (2010) in Mauritius and Mumbai, India respectively. In the Mauritian study, Jauhangeer (2006) conducted a cohort study that investigated the clinical and microbiological profile of patients with infected diabetic foot ulcers. In line with the aim of the Mauritian study, Jauhangeer (2006) further investigated diabetic foot ulcer patients' resistance to infections by studying single nucleotide polymorphisms (SNP) in host *defensin* genes; which encode broad spectrum host immune defence molecules, defensins, responsible for attacking invading microorganisms. Jauhangeer (2006) observed that differences in SNP profiles of *defensin* genes 1 and 2 in diabetics correlated with the frequency of amputations between Mauritians of African and Indian origins. For example, it was observed that Mauritian diabetic patients of African origin were more susceptible to foot infections with 44% amputation rate than those of Indian origin (Jauhangeer, 2006). In addition, Jauhangeer (2006) also optimised molecular techniques such as random amplified polymorphic DNA (RAPD) and conserved segment PCR amplifications

to characterise anaerobes such as *Finnegoldia magna* and other Gram-positive and Gram – negative aerobes in diabetic foot samples.

Dharod (2010) on the other hand studied the microbiological and antibiotic resistance profiles as well as the dominant species in infected diabetic foot ulcers in an Indian cohort study in comparison with a UK cohort. Dharod (2010) investigated bacterial pathogenic mechanisms such as glycan–lectin interactions responsible for cell–cell adherence and biofilm formation in establishing infections. Dharod (2010) optimised as in–house biochemical assay, enzyme linked lectinsorbent assay (ELLA) to characterise glycans such  $\alpha$ -2, 6 sialic acid linked to galactose on the cell surface of *F. magna* using a panel of biotinylated lectins (outlined in Table 2.6, section 2.7) and suggested their involvement in cell–cell attachment in biofilm formation and in establishing infections (Severi *et al.*, 2007).

However, the current study presented here also characterised the microbial profiles of diabetic foot ulcers among the Ghanaian diabetic cohort recruited for the study, and in addition studied the ability of identified bacteria to form biofilm, mechanisms underlying their ability to cause persistent infections and attempted the development of *in vitro* assays as strategies to antagonise pathogenic effects of bacteria in wounds through the inhibition of quorum sensing and biofilm formation. Previous studies in DM with respect to secondary complications such as hypertension and nephropathy and the burden of the disease on the socioeconomic status of Ghanaians have also been reported (Asumanu *et al.*, 2010; Danquah *et al.*, 2012). The operational prevalence of DM in Ghana, 6.3%, established in 2002 provided the basis for revisions as well as formulation and implementation of new health policies in the management of diabetes in Ghana (Amoah, *et al.*, 2002). The prevalence of DM, 22.8% established in the present study, the first to be carried out in a major hospital in Ghana, reveal that little has been done in terms of the management of the disease. The present study therefore supports the idea that, there is the need for the formulation and implementations of stringent policies on early diagnosis, treatment and follow–up programmes in the management of DM in Ghana in line with WHO–predicted insurgence of the disease in developing countries. In addition, prevalence of DFU was estimated as 0.7% which is 5.7 – 38.6 times lower than the global prevalence (Abbott *et al.*, 2002; Bakri *et al.*, 2012; Richard and Schuldiner, 2008; Yazdanpanah *et al.*, 2015). The current “I want my leg back” campaign against amputation at the Diabetes Centre, KATH must be commended for

the decrease in DFU among diabetic patients accessing the centre. The spread of such campaigns across the major health centres in Ghana should be encouraged. Other findings such as the high prevalence of the disease among women (72%) than men (28%) is in conformity with the trend of the disease among people of sub-Saharan African and South Asian descent who reportedly are genetically predisposed to the disease (Chen *et al.*, 2012; Diabetes in the UK, 2012; Gatineau, 2014).

In all 407 bacterial isolates were identified from 356 DFU samples collected during the period of the study. No (strict) anaerobe was isolated as there was no dedicated anaerobic identification system at the site of sample collection and processing. The presence of bacteria (both aerobes and anaerobes), dominant species and their ability to cause pathogenic infections in wounds including diabetic foot ulcers have been well studied (Dharod, 2010; Gardner *et al.*, 2013; Jauhangeer, 2006; Malik *et al.*, 2013; Spichler *et al.*, 2015). As a result, the current work also studied the role of two facultative anaerobes, *Klebsiella pneumoniae* and *Proteus mirabilis* in maintaining the chronicity of diabetic wounds. *K. pneumoniae* and *P. mirabilis* were selected as the main test strains because they were recovered from Wagner's grade 3 (abscess osteitis) and 2 (deep) in-patients' wounds respectively. They also showed resistance to more than 3 antibiotics including ciprofloxacin, ofloxacin, gentamicin, tobramycin and ceftazidime (for *P. mirabilis*), and gentamicin, cefotaxime, amikacin and ceftazidime (for *K. pneumoniae*) respectively. Out of the 407 DFU isolates, 50 of them belonging to the Proteobacteria group were taxonomically classified using the protein-coding nucleotide sequences of their 16S rRNA genes. The phylogenetic analysis of the 50 isolates was important in demonstrating their evolutionary relatedness as either divergent or convergent as illustrated by the phylogenetic tree and its branches and explained in Figure 3.7 and section 3.5. Also, information from the phylogenetic data of some bacteria either within the same or different species or even strains of the same species with high degree of 16S rRNA homology, can provide the basis for the study of virulence genes that are shared by these bacteria. For example, two bacterial species, *P. mirabilis* DFI020 and *K. pneumoniae* DFI034a, and two *K. pneumoniae* strains DFI032 and DFI035 with 100% 16S rRNA homology (located in region C of the phylogenetic tree in Figure 3.7) can be studied to determine whether they belong to the same functional equivalent pathogroups using bioinformatics techniques such as multivariate hierarchical clustering described by Dowd *et al.*, 2008b). Dowd *et al.*, (2008b) demonstrated that polymicrobial infections may comprise



of specific mixtures of anaerobes and aerobes that can symbiotically act (through quorum sensing) to establish pathogenic biofilm infections (Bowler *et al.*, 2001; Brook, 1987; Mayrand and McBride, 1980; Rostein and Kao, 1988). Also, whole genome sequence mapping can be performed to identify specific genes that encode virulence factors such as toxin production which are common to these bacteria with 100% 16S rRNA homology. The phylogenetic data generated for bacteria with high degree of 16S rRNA homology can also provide the basis for the study of antibiotic resistance genes; which can be acquired by horizontal gene transfer within a bacterial consortium in an environment setting or probably through polymicrobial biofilm infections (Antonova and Hammer, 2011; Fux *et al.*, 2005; Kamilar and Cooper, 2013; Lerat and Moran, 2004; Ochman *et al.*, 2000). It can therefore be suggested that, the characterisation of antibiotic resistance genes, whole-genome sequencing, couple with 16S rRNA homology profiling can enhance the typing of diabetic foot isolates for diagnosis and treatment of wounds, epidemiological surveillance of infectious diseases and the control of the spread of antimicrobial resistance (Köser *et al.*, 2014; Reuter *et al.*, 2013).

The presence of *K. pneumoniae* and *P. mirabilis* in clinical conditions such as pneumonia, urinary tract infections (UTIs) and septicaemia have been studied (Chong *et al.*, 2013; Dowd *et al.*, 2008b; Podschun and Ullmann, 1998). Though *K. pneumoniae* and *P. mirabilis* have also been found in diabetic foot infections their contributions to impaired wound healing have not been fully explained (Gardner *et al.*, 2013; George *et al.*, 2015; Gjødtsbøl *et al.*, 2006; James *et al.*, 2008; Malik *et al.*, 2013; Oates *et al.*, 2012; Rhoads *et al.*, 2012). In Chapter 4 of the present work, the role of *K. pneumoniae* and *P. mirabilis* biofilms in diabetic foot infections was investigated. *K. pneumoniae* and *P. mirabilis* were not only found to be strong biofilm producers at normal *in vitro* growth conditions, but also found to produce persister cells during stationary phase of growth and when challenged with antibiotics at high concentrations. When changes were made *in vitro* to the environmental conditions that support their growth, *K. pneumoniae* biofilm was found to be inhibited at low pH (4), high temperature (42°C) but grew quite well at pH 10 and at 26°C. Although high alkaline pH of up to 8.9 has been recorded in non-healing wounds, pH of 10 which was set as the upper limit for the pH biofilm inhibition experiments was for reference only (Gethin, 2007; Romanelli *et al.*, 1997; Tsukada *et al.*, 1992; Wilson *et al.*, 1979). However, it is noteworthy that, the development of any antimicrobial intervention in the treatment of wounds should

have or attain bioavailability even at pH of 8 or more. This is because certain enzymes such as elastase, plasmin and neutrophil elastase that prevent wound healing have been found to attain peak activity between pH of 8.0 to 8.3 (Greener *et al.*, 2005). Although *P. mirabilis* biofilm on the other hand formed normally at 26°C and pH 10, its growth in Luria–Bertani broth was significantly inhibited when nutrient concentrations were reduced by 2–fold or below. The significant effects of environmental changes in inhibiting *K. pneumoniae* and *P. mirabilis* biofilms can be considered in the development of treatment strategies in eradicating biofilms in infected wounds. For example, low or acidic pH has been found to support chronic wound healing (Milne and Connolly, 2014; Percival *et al.*, 2014). Also, the use of Manuka honey as an antimicrobial agent has been found to induce low pH which negatively affects the growth of bacteria such as *P. mirabilis* (Milne and Connolly, 2014).

In their biofilm states, *K. pneumoniae* and *P. mirabilis* were resistant to ceftazidime and levofloxacin 1280 and 10 times, and 64 and 10 times more than their respective therapeutic concentrations (EUCAST, 2014). Although the individual antibiotic resistance mechanisms *K. pneumoniae* and *P. mirabilis* were not evaluated, data from their antibiogram, their ability to form biofilms coupled with other unknown combinations of antimicrobial resistance mechanisms suggest that they may contribute to the persistence of pathogenic infections. The ability of the test strains used in the current study to form persister cells was investigated using the method described by Keren *et al.*, (2004). *K. pneumoniae* and *P. mirabilis* were found to produce persister cells when challenged by antibiotics and at low levels during stationary growth phase. The ability of the multidrug resistant *K. pneumoniae* and *P. mirabilis* DFU strains to form biofilms and produce persister cells is a worrying scenario which has bad prognosis for wound healing. Perhaps the combinatorial effect of two or more antibiotics or antimicrobial agents may provide a possible treatment regime.

In the study presented here, the *Quasi-Vivo*<sup>®</sup> assay, a continuous flow cell culture system was adapted for the study of time–dependent killing of biofilms grown on cover slip. The purpose of this assay was to mimic *in vivo* conditions in an attempt to develop a strategy for biofilm eradication using predetermined antibiotic concentrations. Though eradication of biofilm was not achieved at the pre–determined concentrations (512 and 5120 µg/mL) of ceftazidime and levofloxacin used, fluorescent–stained biofilms on cover–slips demonstrated the visualisation of live and dead bacterial cells using epifluorescence microscopy. The images from

epifluorescence microscopy enabled the qualitative assessment of the efficacy of the antibiotics used by comparing the number of live and dead stained bacterial cells.

The concluding part of the present work investigated the role of bacterial communication, quorum sensing, in the regulation of biofilm formation by *K. pneumoniae* and *P. mirabilis*. Genomic studies have demonstrated the presence of interspecies AI-2 quorum sensing system in some Gram-negative and Gram-positive bacteria which was confirmed to be present in *K. pneumoniae* and *P. mirabilis* DFU isolates by PCR amplification of the *luxS* gene. Multiple sequence alignment of the *luxS* genes in *K. pneumoniae*, *P. mirabilis* and in *Vibrio harveyi*, where it was first discovered, showed high degree of homology. The current study further demonstrated that exogenously produced AI-2 from *K. pneumoniae* and *P. mirabilis* cell-free culture fluids could be used to induce biofilm formation in *V. harveyi luxS* mutant strain. Interspecies QS was also confirmed using the bioluminescence assay through the induction of light production in the *V. harveyi* reference strain used in the current study. The bioluminescence study presented here and other previous studies suggest that, *V. harveyi* and other luminous *Vibrio* species such as *V. fischeri* can be used as biosensor reporter strains in the expression of QS-associated genes responsible for interspecies AI-2 bacterial communications among Gram-negative bacteria explained in section 5.7 (Bassler, 2002; Meighen, 1991; Nealson and Hastings, 1979). Brackman *et al.* (2011) have demonstrated that, the inhibition of quorum sensing using quorum sensing inhibitors such as hamamelitannin, baicalin hydrate and cinnamaldehyde can increase the bacterial biofilms susceptibility to antibiotic treatment. This observation by Brackman *et al.* (2011) was confirmed in the current study as the effective combination of baicalin hydrate, cinnamaldehyde and 2(5H)-furanone and the antibiotics, ceftazidime and levofloxacin, significantly inhibited *K. pneumoniae* and *P. mirabilis* biofilms compared to antibiotics only assays (section 5.7).

To determine if *K. pneumoniae* and *P. mirabilis* produce other QS molecules apart from AI-2, AHL production and detection using the *A. tumefaciens* NCIMB 14543 biosensor reporter assay (also known as the cross-feeding assay as outlined in section 2.6.1.2, Chapter 2) was performed which suggested that both *K. pneumoniae* and *P. mirabilis* produced acyl-homoserine lactones (AHL). The determination of the exact AHLs produced by these 2 DFU isolates was beyond the scope of the present study in terms of budget allocation and time. In a

similar study conducted by Yin *et al.*, (2012), *Agrobacterium tumefaciens* NTL4(pZLR4) and *Escherichia coli* [pSB401] biosensor reporter strains together with high resolution mass spectrometry was used to determine and confirm the production of short and long chained – acylated homoserine lactones, *N*-octanoyl-homoserine lactone and *N*-3-dodecanoyl-L-homoserine lactone by *K. pneumoniae*. In another study by Ngeow *et al.* (2013), *K. pneumoniae* was found to produce a short chain acyl-homoserine lactones, *N*-hexanoyl-homoserine lactone (C6-HSL) using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. On the other hand, the production of AHL by *P. mirabilis* has not yet been discovered. However, a study conducted by Stankowska *et al.* (2012) demonstrated that exogenous addition of AHL such as *N*-butanoyl-homoserine lactone increased the production of exopolysaccharide during *P. mirabilis* 018 biofilm formation. The possible production of AHL by the DFU *P. mirabilis* strain used in the present study can be reconsidered and confirmed using techniques such as mass spectrometry and thin layer chromatography.

Glycan-lectin assays performed to determine the synthesis of exopolysaccharides and other cell-surface glycans suggested the production of polysaccharides during biofilm formation by *K. pneumoniae* and *P. mirabilis* DFU isolates. A panel of plant biotinylated lectins detected the presence of mannose, galactose, glucose and *N*-acetyl-D-galactosamine monomers in cell-surface glycans. The glycan-lectin results imply that the assay can be adapted for screening of cell-surface sugars for diagnostic purposes. It has previously been demonstrated that certain carbohydrate monomers such as L-rhamnose and D-fucose are strong inhibitors of bacterial coaggregation while galactosides such as methyl galactoside, D-galactose, lactose, and  $\alpha$ -methyl galactoside are weak coaggregation inhibitors (Weiss *et al.*, 1987). In a separate study, Ruhl *et al.* (2014) also investigated the interspecies adhesion between coaggregating dental plaque isolates such as type 2 fimbrial adhesin-bearing *Actinomyces naeslundii* and receptor polysaccharide-bearing *Streptococcus oralis* using an *in vitro* solid-phase fluorescence-based assay. Specific fimbrial adhesin-receptor polysaccharide mediated interaction was detected between the two coadhesive partners which were also present in other adhesin and/ or receptor bearing strains of *Neisseria pharyngitis*, *Rothia dentocariosa*, and *Kingella oralis* (Ruhl *et al.*, 2014). Nonetheless, it can be suggested that glycan-lectin profiles of bacterial coaggregating partners can provide useful data for the study and characterisation of bacterial cell surface appendages involved in mixed species biofilms. Data

from glycan–lectin and coaggregation assays can also direct the strategic design of antagonists that can inhibit protein–sugar binding to control biofilm formation in Gram–negative bacteria (Bales *et al.*, 2013; Vu *et al.*, 2009).

Current wound dressings on the market have been designed to target planktonic bacteria rather than biofilms in wounds. In the work presented here, 3 wound dressings Acticoat<sup>®</sup> (ACT), Medihoney<sup>™</sup> Apinate (MDA) and Silvercel<sup>®</sup> (SIL) were able to inhibit *K. pneumoniae* and *P. mirabilis* biofilms by 22 to 61.7% using a 6–well plate assay. In a second method, planktonic or sessile growth of the same bacteria on a standard agar (Mueller–Hinton) or poloxamer gel were found to be resistant to ACT, MDA and SIL. Poloxamer gel biofilm models which were first described by Gilbert *et al.* (1998) are known to create or mimic the characteristic biofilm environment with localised high cell densities, growth rates and oxygen gradients from the top to the bottom of the biofilm. Biofilms grown on poloxamer gels have also been demonstrated to exhibit more clinically relevant biofilm phenotypes that express outer membrane proteins between 78 and 87 kilo Daltons, and show enhanced resistance to biocide (Gilbert *et al.*, 1998; Wirtanen *et al.*, 1998). In both assays, bacterial cell suspension of 10<sup>8</sup> CFU/mL was challenged by a 2–cm–diameter wound dressing. Two or more methods have been described for the determination of the inhibitory effects of wound dressings (Hill *et al.*, 2010; Percival *et al.*, 2007; Wright *et al.*, 1998). The disparity in the methods used in the present study, despite at least 3 replicates of the assay performed, suggests that differences in techniques used in the assays may affect the results. Optimisation of both assays to include similar materials and conditions may help to redress this disparity.

The inhibition of QS in *K. pneumoniae* and *P. mirabilis* biofilm formation by 2(5H)–furanone and CIN in the present study suggests that quorum sensing inhibitors can increase the susceptibility of bacteria that colonise diabetic foot ulcers to killing. The subsequent combination of the antibiotics, ceftazidime and levofloxacin, with QS inhibitors, BH, CIN and 2(5H)–furanone, or with antimicrobial peptides, PMB and PMBN in synergy assays significantly inhibited *K. pneumoniae* and *P. mirabilis* biofilm formation and in effect suggest that, effective combination of antimicrobial and antibiofilm agents may provide alternate treatment regime that can significantly inhibit the growth diabetic foot isolates.

For the past decade, attempts are being made to combat persister cells through the combination of antibiotics, antimicrobials and other molecules such as enzymes (Brötz–Oesterhelt *et al.*, 2005; Conlon *et al.*, 2013). Brötz–Oesterhelt *et al.* (2005) have documented the successful eradication of Gram–positive bacterial cells with the caseinolytic protease P, ClpP; a subunit of protease enzyme Clp. Clp is ubiquitous in all bacteria and can be regulated for the breakdown of some specific proteins (Gerdes and Ingmer, 2013). The enzyme consists of a proteolytic subunit ClpP which has two heptameric rings with small central pores which lead to the central proteolytic chamber where peptide chains are strung and degraded after they are unfolded by the Clp ATPase regulatory enzymes (Frees *et al.*, 2007). ClpP was also found to be capable of uncontrolled proteolytic degradation when activated by a peptide antibiotic called acyldepsipeptide (ADEP) (Conlan *et al.*, 2013). ADEPs are a new class of antibiotic peptides isolated through fermentation from broth cultures of *Streptococcus hawaiiensis* NRRL 15010 (Brötz–Oesterhelt *et al.*, 2005). They are effective against multidrug resistant pathogens and act by dysregulating ClpP in a process that leads to uncontrolled protein degradation and subsequent cell death.

A recent study has also confirmed the successful eradication of persister cells in chronic biofilm infections using ADEP4–activated ClpP which degraded over 400 proteins compelling bacterial cells to eventually undergo autolysis (Conlon *et al.*, 2013). It was shown that ADEP4 in the presence of conventional rifampicin completely eradicated persister cells in a *S. aureus* biofilm. On the other hand, less success has been achieved with Gram–negative bacteria. This is because in Gram–negative bacteria, an outer membrane permeabilizer such as polymyxin B nonapeptide (PMBN) a derivative of polymyxin B (PMB), is required before ADEP can activate ClpP in the bacterial cell (Tsubery *et al.*, 2002). PMB binds to the negatively charged lipopolysaccharide (LPS) on the outer membrane which increases membrane permeability by causing membrane damage (Tran *et al.*, 2005). However, unlike ADEP, PMB has been found to be nephrotoxic and neurotoxic and therefore discontinued in clinical practice (Falagas *et al.*, 2006). In the current study, almost all the isolates were Gram–negative and it will be interesting to study the effect of other ADEP and its derivatives and activated ClpP in the presence of other cell membrane permeabilisers.

*In vitro* antibiotic assays suggest that high dosage of antibiotics can successfully eradicate biofilms. However, the application of this in clinical practice can lead to the development of

secondary antibiotic resistance in patients and cause cytotoxicity (Bordi and Bentzmann, 2011). Diabetic foot ulcers can also be treated by cleaning and sterilising the wound with antiseptics such as hydrogen peroxide, 70% ethanol and povidine-iodine. Unfortunately, bacteria have developed resistance to these sterilising agents. Low concentrations of H<sub>2</sub>O<sub>2</sub> (0.25% – 1%) which are recommended for clinical use have been found to increase bacterial tolerance and resistance as explained earlier (Cochran *et al.*, 2000; Elkins *et al.*, 1999). Other treatment options include surgical debridement and amputation (Fisher *et al.*, 2010; Davis *et al.*, 2006; Frykberg, 2000). The different debridement methods include enzymatic, water-jet powered and ultrasound techniques. Debridement can be effective when it is considered together with antimicrobial chemotherapy. It has been found that surgical debridement procedures affect quorum sensing within biofilms leading to reduction in coordinated virulence (Davis *et al.*, 2006).

Diabetic foot ulcers can be prevented in diabetics who adhere to a stringent lifestyle that includes avoiding the use of flip-flops, walking bare foot, smoking, and drinking alcohol. Tobacco smoke has been found to be toxic to the pancreas and contributes to the development of chronic pancreatic cancer (Lynch *et al.*, 2009). Alcohol on the hand contributes to the development and aggravation of diabetes in 3 ways namely: reducing the body's sensitivity to insulin leading to increase blood glucose levels, increasing risk of pancreatitis which results in diabetes, and increasing the risk of obesity through the addition of excess calories to the body (Shah, 1987; NHS Choices websites). Diabetics can also develop foot ulcers from injured foot when drunk. Diabetic patients who regularly take their medications to control their glucose levels will reduce the risk of having a foot ulcer. In Ghana, the development of ulcer among diabetics is largely due to treatment default, unhealthy eating and alcoholism (this was established through a personal conversation with Prof Benjamin A. Eghan, and staff at the Diabetes Centre, KATH, Kumasi, Ghana).

This study has demonstrated the varying efficacies of conventional antibiotics in inhibiting and disrupting biofilms. From this and other previous studies, it is imperative to mention that the future of biofilm eradication probably lies in the effective combination of antibiotics that will target all the different stages of biofilm formation. However, due to ischaemic conditions in DFUs, active metabolites from orally administered antibiotics most often do not reach their target sites in order to effect treatment. Therefore, effective formulation of antimicrobial

treatment should consider topical application to achieve effective treatment. Nevertheless, the complete treatment of chronic wounds should consider the treatment of the main underlying cause as well as accompanying complications.

## 6.2 Future work and recommendation

The present work investigated the ability of *K. pneumoniae* and *P. mirabilis* to produce persister cells at different phases of growth and after antibiotic challenge. Further studies are needed to investigate the molecular mechanisms by which they form persister cells. Currently such studies have been successful with *E. coli* (Lewis, 2010; Maisonneuve and Gerdes, 2014; Moyed and Bertrand, 1983). A confirmation of persister cells formation with a molecular genetic basis will increase the understanding of the role of persistence in chronic wounds.

Some schools of thought have suggested that multiprotein families of conserved proteins should be the basis for phylogenetic analysis rather than the use of small-subunit rRNA sequences to provide a more robust taxonomical classification and a comprehensive analysis of evolutionary relatedness of bacterial families (Gregoretto *et al.*, 2004; Williams *et al.*, 2010; Wolf *et al.*, 2001). Reconstruction of the phylogenies of all 50 DFU isolates presented in the current study will be necessary to fully understand the evolutionary relatedness of some members of the Proteobacteria group.

In this study, *K. pneumoniae* and *P. mirabilis* were found to be AHL producers using *A. tumefaciens* NCIMB 14543 as a biosensor reporter in the cross-feeding assay. The confirmation of the exact AHLs produced by these DFU isolates may provide further understanding in their use of QS systems either for virulence expression, pathogenicity or acquisition of antibiotic resistance. The characterisation of AHLs produced by *K. pneumoniae* and *P. mirabilis* in this study can also provide potential targets for complete inhibition of biofilms through QS inhibition.

The use of *gyrase B* PCR amplification of both wild-type and persister cells of *K. pneumoniae* and *P. mirabilis* as a differential test of persister cell formation must be supported by a series of tests that will allow the cloning and expression of highly conserved protein or a family of proteins whose expressions are directly or indirectly regulated during biofilm formation.



A recent study by Carvalhais *et al.* (2015) characterised 19 intracellular proteins of *S. epidermidis* that were expressed during an induced dormancy in a human–bacteria immune interaction. They have suggested that immune–proteomic approaches may provide fresh insight into the expression of possible biofilm markers by bacteria that respond to either host immune serum or externally administered antimicrobial agents. A similar study by Conlon *et al.* (2013) used an antimicrobial peptide, acyldepsipeptide (ADEP4) to dysregulate intracellular proteins, ClpP, leading to the degradation of more than 400 proteins and subsequent autolysis.

The initial plan for this study was to collate demographic data as well as some clinical history of participants in order to establish the complete clinical presentation of diabetic patients recruited for the study. Though plans were far advanced in this direction, the practicality of these plans was not realised as some participants were willing and others were not. The other shortcomings were due to the fact that staff recruited at the site of sample collection were very busy and could not fit taking participants’ details into their working schedules. A follow–up on the present work should consider recruiting dedicated staffs that are not necessarily part of the clinical teams at the site of sample collection.

Though this study has demonstrated the importance of clinical biofilms produced by aerobes and facultative anaerobes, the demonstration of biofilm formation by anaerobes isolated from the Ghanaian cohort study would have provided basis for comparison and more understanding into the role of anaerobes in impaired wound healing. It is recommended that a follow–up on the work presented here should consider equipping the site of sample collection with a dedicated anaerobic bacterial isolation unit.

The characterisation of AHLs produced by *K. pneumoniae* and *P. mirabilis* in this study can provide potential targets for complete inhibition of biofilms through QS inhibition. Future work should consider the complete characterisation of these AHL molecules by either gas chromatography–mass spectrometry or thin layer chromatography to determine the length of their respective acyl chains (either short or long). The determination of the length of the acyl chains will provide more information on their functionality (i.e., their ability to bind to a transcriptional regulator and elicit quorum sensing response). Finally, data obtained from the determination of FIC indices can be experimented on animal models to determine effective

additive and synergy effects which can be recommended for the formulation of topical treatment for DFU.

### 6.3 Conclusions

The work presented here is the first to establish the prevalence of diabetes mellitus and diabetic foot ulcer among patients attending the Diabetes Centre, Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana since 2002 when Amoah *et al.* (2002) established national prevalence. A total of 407 bacterial isolates were recovered from 356 diabetic foot ulcers which were sampled from January 2011 to December 2014. This work has also provided the taxonomic classification of 50 out of the 407 DFU isolates based on the phylogeny of their 16S rRNA genes to demonstrate their diversity. Data generated from the taxonomic classification together with the antibiotic susceptibility patterns of these isolates can serve a guide in the selection of treatment options for diabetic foot management at the KATH.

This study has provided evidence that, DFU isolate can form biofilms. Two representative DFU isolates, *Klebsiella pneumoniae* and *Proteus mirabilis* further showed their ability to produce persister cells and were resistant and/or tolerant to antibiotic concentrations as high as 1280 times their therapeutic MIC. *K. pneumoniae* and *P. mirabilis* also showed their ability to communicate with other bacterial species (*V. harveyi*) through AI-2-mediated quorum sensing. In addition, *K. pneumoniae* and *P. mirabilis* were found to produce AHLs which were not characterised in this study. It can therefore be said that in addition to *E. coli*, *Staphylococcus aureus* and *P. aeruginosa*, *K. pneumoniae* and *P. mirabilis* can now serve as model strains for future research into biofilm, QS and persister cell formation.

A dedicated continuous flow cell culture system called *Quasi-Vivo*<sup>®</sup> system produced by Kirkstall Ltd, UK was adapted to demonstrate time-dependent killing of biofilms. The resultant antibiotic effect was determined through the visualisation of live/dead cells using epifluorescence microscopy. This was the first time the equipment had been used to study bacterial cells.

This study also demonstrated through antibiotic-quorum sensing inhibitor combination assays that, the synergy between antibiotics and other antimicrobial agents have the potential to significantly inhibit DFUs colonised by *K. pneumoniae* and *P. mirabilis*. It was also shown

that the individual antimicrobial efficacy of some wound dressings currently in use to treat DFU may be ineffective against some wound isolates such as *K. pneumoniae* and *P. mirabilis*. Nonetheless, the combined efficacy of wound dressings and antibiotics significantly inhibited both planktonic and quasi-biofilms.

In summary, this thesis has provided evidence that DFUS provides potential environments that support the biofilm growth of pathogenic and opportunistic bacteria and may provide the platform for the exchange of multidrug resistance genetic materials. The ability of these DFU isolates to resist and/or tolerate antibiotic concentrations more than 1000 times their therapeutic range poses a threat to their treatment and public health. However, the combinatorial (additive) effect and synergy between antibiotics and other antimicrobial agents (derived from natural products) such as cinnamaldehyde, baicalin hydrate, 2(5H)-furanone, polymyxin B and polymyxin B nonapeptide provide reassurance that they are treatable.

# Chapter 7

## 7.1 APPENDICES

### 7.1.1 Appendix A

#### Recipes for media and solutions

**Table A 1** *V. harveyi* growth media and their compositions

<b>Media</b>	<b>Amount</b>	<b>Media</b>	<b>Amount</b>
<b>L–marine agar</b>	<b>Per 1 Litre</b>	<b>Sea–water agar</b>	<b>Per 1 Litre</b>
Tryptone	10 g	Sea water	750 mL
Yeast extract	5 g	Agar	20 g
NaCl	20 g	Peptone	10 g
Agar*	15 g	Beef extract	10 g
Distilled water	Add up to 1 litre	Distilled water	Add up to 1 litre

**Table A 2** Composition of ATCC 2746 Autoinducer Bioassay (AB) medium

<b>Component A (Base medium)</b>		<b>Component B (Stock solutions)</b>	
NaCl	17.5 g	1 M KH <sub>2</sub> PO <sub>4</sub>	10 mL
MgSO <sub>4</sub>	12.3 g	0.1 M L–arginine	10 mL
Casamino acids	2.0 g	Glycerol (50%)	10 mL
Deionised water	970 mL		

NB – The base medium was prepared and the pH of the solution brought to 7.5 with 3 M NaOH and sterilised by autoclaving at 121°C for 15 minutes. The stocks solutions were also prepared separately and sterilised by autoclaving. To make 1 litre of the final AB medium 970 mL of the base medium and 10 mL each of the stock solutions were added.

**Table A 3** Composition of BM medium

<b>Media</b>	<b>Amount (g/L)</b>
Proteose peptone	10
Trypticase peptone	5
Yeast extract	5
KCl	2.5
Haemin	0.005
Vitamin K <sub>1</sub>	0.001
L-Cysteine HCL	0.5
Glucose	10

**Table A 4** Coaggregation buffer (1 Litre of solution at pH 8.0)

<b>Compound</b>	<b>Amount</b>
CaCl <sub>2</sub>	0.1 mM
MgCl <sub>2</sub>	0.1 M
NaCl	0.15 M
Tris-HCl	1 mM

**Table A 5** 50X TAE buffer (1 Litre of solution in deionised water)

<b>Compound</b>	<b>Amount</b>
Tris base (Mw – 121g/mol)	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA	100 mL

Note: 0.5 M EDTA was prepared at pH 8.0

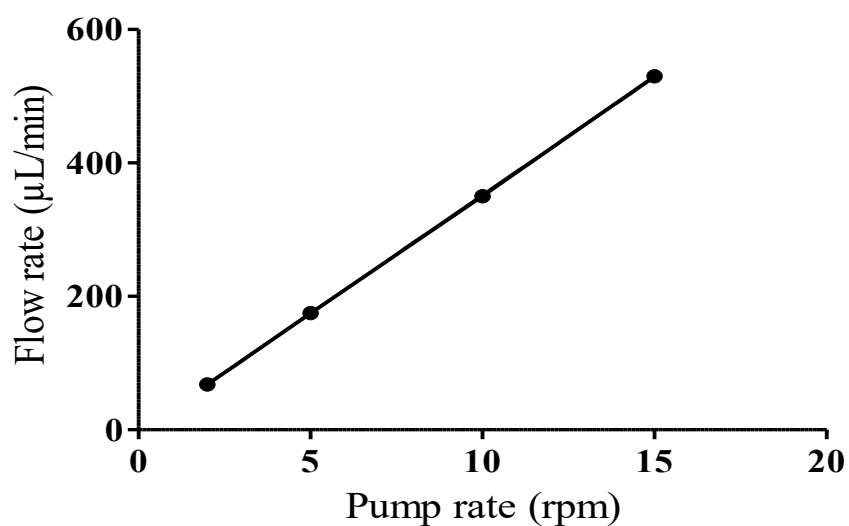
**Table A 6** 10X phosphate buffered saline (PBS) in 1 Litre of solution

Compound	Amount
NaCl	58.44
KCl	74.55
Na <sub>2</sub> HPO <sub>4</sub>	141.96
KH <sub>2</sub> PO <sub>4</sub>	136.09

Note: Add deionised water to bring to 1 Litre and autoclave to sterilise.

### 7.1.2 Appendix B

#### Calibration of *Quasi-Vivo*<sup>®</sup> QV500 chamber system



**Figure B 1** Calibration curve for the QV500 chamber system for the 3/32" diameter tubing

### 7.1.3 MBEC™ HTP assay protocol (Harrison *et al.*, 2005)

#### PROTOCOL

High-throughput (HTP) metal susceptibility testing of microbial biofilms using the

MBEC™-HTP assay

Last revised by Joe J. Harrison, February 11, 2005

Disclaimer: Mention of trade names or commercial products in this protocol is solely for the purpose of providing specific information and does not imply endorsement by the authors.

An overview of all steps in this protocol is provided in supplementary Figure S1.

This protocol has been developed for use with Nunc Brand, flat bottom, 96-well microtiter plates. These microplates have a maximum volume of 300 µl per well. The medium and buffer volumes listed here may need to be adjusted for different brands of microtiter plates.

#### A. Inoculating the MBEC™-HTP assay plate

1. From the cryogenic stock (at  $-70^{\circ}\text{C}$ ), streak out a first sub-culture of the desired bacterial strain on an appropriate agar plate. Incubate for up to 24 h at the optimum growth temperature of the microorganism. The first sub-culture may be wrapped with Parafilm™ and stored at  $4^{\circ}\text{C}$  for up to 14 days.
2. Check the first sub-culture for purity (i.e. only single colony morphology should be present on the plate).
3. From the first sub-culture, streak out a second sub-culture on an appropriate agar plate. Incubate for up to 24 hours at optimum growth temperature of the microorganism. The second sub-culture must be used within 18 to 30 h starting from the time it was first placed in the incubator.
4. Verify the purity of the second sub-culture.

**IMPORTANT: DO NOT GROW THE SUB-CULTURES ON MEDIA CONTAINING A SELECTIVE AGENT. ANTIBIOTICS AND OTHER ANTIMICROBIALS MAY TRIGGER AN ADAPTIVE STRESS RESPONSE IN BACTERIA. THIS MAY RESULT IN AN ABERRANT SUSCEPTIBILITY DETERMINATION.**

The following steps must be carried out in a biological safety cabinet (BSC):



5. Obtain a sterile 96-well microtiter plate. For each MBEC™-HTP assay, fill 4 'columns' of the microtitre plate from 'rows' A to F with 180 µl of a physiological saline solution (ie. 0.9% saline or phosphate buffered saline).

6. Put 1.5 ml (plus 1.0 ml for each additional MBEC™ device being inoculated at the same time) of the desired broth growth medium into a sterile glass test tube.

7. Using a sterile cotton swab, collect the bacterial colonies on the surface of the second agar subculture. Cover the tip of the cotton swab with a thin layer of bacteria.

8. Dip the cotton swab into the broth to suspend the bacteria. The goal is to create a suspension that matches a 1.0 McFarland standard (i.e.  $3.0 \times 10^8$  CFU/ml). Be careful not to get 'clumps' of bacteria in the solution.

9. Repeat steps 6 and 7 as many times as required to match the optical standard. Page 2

10. Put 29 ml of the appropriate broth growth medium into a sterile 50 ml polypropylene tube. To this, add 1.0 ml of the 1.0 McFarland standard bacterial suspension. This 30-fold dilution of the 1.0 McFarland standard (i.e.  $1.0 \times 10^7$  CFU/ml) serves as the inoculum for the MBEC™ device.

11. Open the sterile package of the MBEC™-HTP assay. Put 22.0 ml of the inoculum into the corrugated trough of the MBEC™ device. Place the peg lid onto the trough. Label the device appropriately.

12. Place the device on a rocking table in a humidified incubator at the appropriate temperature. The channels in the trough of the MBEC™ device must be positioned parallel to the direction of motion.

**IT IS CRITICAL THAT THE ANGLE OF THE ROCKING TABLE BE SET BETWEEN 9° AND 16° OF INCLINATION. THIS MOTION MUST BE SYMMETRICAL.**

13. Place a 20 µl aliquot of the inoculum in 'row' A of each of 4 'columns' in the microtitre plate set up in step 5. Serially dilute the inoculum in 10-fold increments along the length of the microtiter plate.

14. Spot plate the serial 10-fold dilutions of the inoculum from 10-6 to 10-1 on an appropriately labelled series of agar plates. Incubate the spot plates for an appropriate period of time and score for growth. These plates are controls used to verify the starting cell number in the inoculum.

B. Setting up the 'challenge' plate

1. Get a brand new, sterile microtitre plate and open it in the laminar flow hood. DO NOT USE RECYCLED MICROTITRE PLATES FOR SETTING UP CHALLENGE PLATES. For example, the Anprolene (ethylene dioxide) gas sterilization method used to recycle the microtitre plates may discolour the plastic, and this may affect optical density measurements obtained using the microtitre plate reader.

2. Setup a working solution of metal cation or oxyanion in the appropriate growth medium. Do not dilute the growth medium by more than 20% (ie. no more than 1 part metal solution per 4 parts of growth medium). The working solution of the metal should be made at a concentration equal to the highest concentration to be tested in the challenge plate.

3. Add 200  $\mu$ l of growth medium to 'column' 1 and 'column' 12 of the challenge plate. These will serve as sterility and growth controls, respectively.

4. Add 100  $\mu$ l of growth medium to 'columns' 3 to 11 of the microtitre plate.

5. Add 200  $\mu$ l of the working solution to 'column' 2 of the microtitre plate.

6. Add 100  $\mu$ l of the working solution to 'column' 3 and 'column' 4 of the microtitre plate.

7. Using the multichannel micropipette, mix the contents of 'column' 4 by pipetting up and down. After mixing, transfer 100  $\mu$ l from the wells in 'column' 4 to the corresponding wells in 'column' 5.

8. Mix and transfer 100  $\mu$ l from 'column' 5 to 'column' 6. Serially repeat this mix and transfer process down the length of the microtitre plate until reaching 'column' 11.

9. Mix the contents of column 11 up and down. Extract 100  $\mu$ l from each well in 'column' 11 and discard.

10. Add 100  $\mu$ l of growth media to the wells in 'columns' 4 through 11.

11. Replace the lid on the challenge plate. Gently tap the plate to facilitate mixing of the metals and media.

DO NOT PREPARE CHALLENGE PLATES MORE THAN 60 MINUTES PRIOR TO USE. METALS CAN

SPONTANEOUSLY REACT WITH MEDIA TO FORM REDUCTION PRODUCTS WITH ALTERED BIOLOGICAL TOXICITY.

C. Exposing the biofilms

1. Setup a sterile microtitre plate with 200  $\mu$ l of physiological saline solution in every well. This plate will be used to rinse the pegs to remove loosely adherent planktonic cells from the biofilm (this is termed a 'rinse plate').

2. Setup a sterile microtitre plate with 200  $\mu$ l of physiological saline solution in 4 'columns' of row A for each MBEC™ device inoculated (ie. 1 microtitre plate is required for every 3 MBEC™ devices). Fill rows B to F with 180  $\mu$ l of physiological saline solution. In a second microtitre plate, fill 4 'columns' from rows A to H with 180  $\mu$ l of physiological saline solution for each MBEC™ device inoculated. The first microtitre plate will be used to do serial dilutions of biofilm cultures, the second will be used to check the growth of planktonic cells in the trough of the MBEC™ device.

3. Following the desired period of incubation, remove the MBEC™-HTP device from the rocker and into the laminar flow hood. Remove the peg lid from the trough and submerge the pegs in the wells of the rinse plate. Let the rinse plate sit for 1 to 2 minutes while performing step 4 below.

4. Use a micropipette to transfer 20  $\mu$ l of the planktonic culture (in the trough of the MBEC™ device) into the 180  $\mu$ l of saline in row 'A' of the latter plate set up in step 2 (immediately above). Repeat this three more times for a total of  $4 \times 20 \mu$ l aliquots.

5. Take the remainder of the planktonic culture and discard it in a solution of dilute 5% bleach. Allow a minimum of 25 minutes to completely eradicate the culture. Immediately discard the trough in the autoclave garbage.

6. In the laminar flow hood, dip a pair of pliers into 95% ethanol. Flame the pliers using the ethanol lamp in the hood.

**CAUTION: DO NOT LIGHT THE ETHANOL LAMP AND DO NOT FLAME THE PLIERS BEFORE YOUR GLOVES HAVE DRIED FOLLOWING DISINFECTION USING 70% ETHANOL.**

7. Using the flamed pliers, break off pegs A1, C1, E1 and G1 from the lid of the MBEC™ device and immerse them in the 200  $\mu$ l of saline in row A (and each in a different 'column') of the first plate setup in step 2.

8. Using the flamed pliers, break off pegs B1, D1, F1 and H1 and discard.

9. Insert the peg lid of the MBEC™-HTP device into the challenge plate. Place the challenge plate in the same incubator where the biofilms were formed (ie. the incubator that houses the rocking table).

10. Place the microtitre plate containing the broken pegs in the tray of the water table sonicator (Aquasonic). Sonicate on the setting 'high' for 5 minutes.

11. Serially dilute 20 µl aliquots of the planktonic cultures (from step 4) in the wells of the corresponding microtitre plate. Once sonication is complete, repeat this serial dilution process with the biofilm cultures.

12. Spot plate the serial 10-fold dilutions of the planktonic and biofilm cultures from 10<sup>-8</sup> to 10<sup>-3</sup> and 10<sup>-5</sup> to 10<sup>0</sup> on an appropriately labelled series of agar plates. Incubate the spot plates for an appropriate period of time and score for growth.

#### D. Neutralization plates, recovery media, MBC and MBEC determinations

Neutralization is a two-part procedure. First, an inorganic agent or a chelator is used to precipitate, coordinate, or reduce the metal to a less biologically toxic species. Second, the neutralized cultures are spot plated onto rich agar media. This latter step allows the diffusion of metals into the rich agar media (where they may be coordinated or precipitated) whilst bacteria remain on top where they may recover.

Neutralizing agents should be prepared as stock solutions in the range of 0.1 to 0.5 M each. The stock solutions should be syringe filtered and stored at -20°C until use.

1. Add the appropriate neutralizing agent (in a quantity to obtain the desired concentration) to 25 ml of rich media (ex. Luria-Bertani media, Tryptic Soy Broth, etc.). 20 ml of recovery medium will be required for each MBEC<sup>TM</sup>-HTP assay used. Add 200 µl of this recovery medium to each well of a brand new, 96-well microtitre plate. This plate is termed the 'recovery plate'.

2. Add the appropriate neutralizing agent (in a quantity to obtain 5 times the desired concentration) to 5ml of physiological saline solution. 2.0 ml of 5 × neutralizing solution will be required for each MBEC<sup>TM</sup>-HTP assay used. Add 10 µl of this neutralizing solution to each well of a (preferably recycled) 96-well microtitre plate. This plate is termed the 'neutralizing plate'.

3. Prepare 2 rinse plates for every MBEC<sup>TM</sup>-HTP assay used (as described in part C, step 1).

4. Remove the challenge plate from the incubator and into the laminar flow hood. Remove the peg lid and immerse in the pegs in the physiological saline of a rinse plate. Cover the challenge plate with the sterile lid of the rinse plate. The challenge plate now contains the planktonic cultures that will be used for MIC and MBC determinations. Label the planktonic cultures appropriately.

5. After approximately 1 min, transfer the peg lid from the first rinse plate into the second rinse plate. While the rinse steps are in progress, proceed with step 6.

6. Using the multichannel pipette, transfer 40 µl of the planktonic cultures from the wells of the challenge plate to the corresponding wells of the neutralizing plate. Allow a minimum of 15 minutes for the neutralization reaction to occur before spot plating.

7. Transfer the peg lid from the second rinse plate into the recovery plate setup in part D, step 1.

Transfer the recovery plate (containing the pegs of the MBEC™ device into the tray of the water table sonicator. Sonicate on high for 5 min.

8. After sonication, remove the peg lid from the recovery plate and replace the original lid of the microtitre plate. Note any reduction or colour changes to the biofilms on the pegs. The lid of the MBEC™ device may now be discarded.

9. Transfer 40 µl from each well of the recovery plate into the corresponding well of a sterile (preferably recycled) 96-well microtitre plate. Place the recovery plate in the same incubator used to form the biofilms and incubate a minimum of 48 h.

10. On an appropriately labelled stack of agar plates, spot 20 µl aliquots of the neutralized planktonic and biofilm cultures (use the aliquots of recovery media prepared in step 9 above, do not spot plate directly from the recovery plate). Incubate at an optimal growth temperature for a minimum of 48 h before scoring qualitatively for growth.

#### Log-Killing and Viable Cell Counts

Instead of preparing neutralization plates as outlined above, transfer planktonic cultures into serial dilution microtitre plates that contain 180 µl of physiological saline solution in each well of rows B to H, and 10 µl of the 5 × neutralizing solution in row A. Serially dilute 20 µl using the multichannel pipette.

Similarly, for biofilm cultures, add 40 µl of the recovery media (containing the sonicated biofilms) from the recovery plate to row A of a serial dilution plate containing 180 µl of physiological saline solution in each well of rows B to F. Serially dilute 20 µl using the multichannel pipette.

Spot plate biofilm and planktonic cultures (which have been serially diluted) on appropriately labelled agar plates. Incubate for a minimum of 48 h.

To calculate log-kill, use the following formula:

$$\text{Log-kill} = \log_{10} (\text{initial cfu/ml}) - \log_{10} (\text{remaining cfu/ml after exposure})$$

To calculate percent kill, use the following formula:

$$\% \text{ kill} = 100 - \{[(\text{initial cfu/ml}) \times (\text{remaining cfu/ml}) / (\text{initial cfu/ml})] \times 100\}$$

#### Collecting Data

1. MIC values are obtained by reading the optical density of the challenge plate at 650 nm (OD<sub>650</sub>) 48 to 72 h after the pegs have been removed from the challenge media.

2. MBC and MBEC values are determined by +/- scoring of growth on the spot plates after a minimum of 48 h incubation. (Note that after this period of time, scoring is not a time sensitive operation).

Alternatively, if viable cell counts are being determined, enumerate the bacteria growing on the spot plates.

3. MBEC values are redundantly determined by reading the OD<sub>650</sub> of the recovery plate on the microtitre plate reader after 48 h incubation.

#### Abbreviations used

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MBEC = minimum biofilm eradication concentration; HTP = high-throughput; MBEC<sup>TM</sup> = the MBEC<sup>TM</sup>-device.

**Table B 1** Synergistic effects of PMB and CAZ/LEV in the inhibition and eradication of *K. pneumoniae* and *P. mirabilis* biofilms

Bacterial strain	Antibiotic	Log <sub>10</sub> reduction							
		MIC/PI <sup>a</sup>		MBEC/PR <sup>a</sup>		MIC/PI <sup>b</sup>		MBEC/PR <sup>b</sup>	
<i>K. pneumoniae</i>	Ceftazidime	40	90	640	50	20	90	640	60
	Levofloxacin	40	> 90	640	> 50	10	> 90	640	> 70
<i>P. mirabilis</i>	Ceftazidime	10	70	640	50	20	70	320	60
	Levofloxacin	10	> 90	640	> 50	10	> 95	320	> 60

<sup>a</sup> – MIC/PI – minimum inhibitory concentration (in µg/mL) and percentage inhibition (%) of biofilm by CAZ and LEV when PMB concentration was 100 µg/mL; MBEC/PR<sup>a</sup> – minimum biofilm eradication concentration (in µg/mL) and percentage reduction (%) of biofilm by antibiotics CAZ and LEV when PMB concentration was 100 µg/mL,

<sup>b</sup> – MIC/PI – minimum inhibitory concentration (in µg/mL) and percentage inhibition (%) of biofilm by CAZ and LEV when PMB concentration was 500 µg/mL; MBEC/PR<sup>b</sup> – minimum biofilm eradication concentration (in µg/mL) and percentage reduction (%) of biofilm by CAZ and LEV when PMB concentration was 500 µg/mL.

**Table B 2** Synergistic effects of PMBN and CAZ/LEV in the inhibition and eradication of *K. pneumoniae* and *P. mirabilis* biofilms

Bacterial strain	Antibiotic	Log <sub>10</sub> reduction							
		MIC/PI <sup>a</sup>		MBEC/PR <sup>a</sup>		MIC/PI <sup>b</sup>		MBEC/PR <sup>b</sup>	
<i>K. pneumoniae</i>	Ceftazidime	40	> 70	640	50	20	> 80	640	> 50
	Levofloxacin	40	60	640	70	10	95	640	> 70
<i>P. mirabilis</i>	Ceftazidime	10	60	640	30	20	> 60	320	> 40
	Levofloxacin	10	> 90	640	50	10	> 95	320	> 60

<sup>a</sup> – MIC/PI – minimum inhibitory concentration (in µg/mL) and percentage inhibition (%) of biofilm by CAZ and LEV when PMBN concentration was 100 µg/mL; MBEC/PR<sup>a</sup> – minimum biofilm eradication concentration (in µg/mL) and percentage reduction (%) of biofilm by antibiotics CAZ and LEV when PMBN concentration was 100 µg/mL,

<sup>b</sup> – MIC/PI – minimum inhibitory concentration (in µg/mL) and percentage inhibition (%) of biofilm by CAZ and LEV when PMBN concentration was 500 µg/mL; MBEC/PR<sup>b</sup> – minimum biofilm eradication concentration (in µg/mL) and percentage reduction (%) of biofilm by CAZ and LEV when PMBN concentration was 500 µg/mL.

### 7.1.4 Appendix C

16S339F DFI036a <i>E. coli</i>	- - - - -	- - - - -
16S339F DFI036b <i>P. stuartii</i>	- - - - -	- - - - -
16S339F DFI037 <i>P. mirabilis</i>	- - - - -	- - - - -
16S339F DFI038 <i>P. aeruginosa</i>	T G A G T T T	T G A T C C T G G C T C A G A T
16S339F DFI001 <i>P. mirabilis</i>	- - - - -	- - - - - T G C C G C G T G
16S339F DFI002 <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI003A <i>C. koseri</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI003B <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI004 <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI005 <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI006 <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI007 <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI008 <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI009 <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI010a <i>E. coli</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI010b <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI011 <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI012 <i>E. coli</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI013 <i>E. coli</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI014 <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI015 <i>E. coli</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI016 <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI017 <i>E. coli</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI018a <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI018b <i>K. variicola</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI019a <i>E. coli</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI019b <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16SV3R DFI020 <i>P. mirabilis</i>	- - - - -	- - - - -
16S339F DFI021a <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI021b <i>K. pneumonia</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI022a <i>E. coli</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI022b <i>K. pneumonia</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI023a <i>K. pneumonia</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI023b <i>P. aeruginosa</i>	- - - - -	- - - - - T G C C G C G T G
16S339F DFI024 <i>E. coli</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI025a <i>P. stuartii</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI025b <i>P. mirabilis</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI026 <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI027 <i>K. pneumoniae</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI028a <i>E. coli</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI030 <i>E. coli</i>	G C C T G A T	G C A G C C A T G C C G C G T G
16S339F DFI031 <i>E. coli</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI032a <i>P. mirabilis</i>	G C C T G A T	G C A G C C ? T G C C G C G T G
16S339F DFI032b <i>K. pneumonia</i>	G C C T G A T	G C A G C C ? T G C C G C G T G

**Figure C 1** Multiple Sequence Alignment of 50 16S rRNA protein-coding sequences aligned using MEGA6 MUSCLE alignment tool. Conserved sequences highlighted in yellow



005 <i>P. mirabilis</i> persister gyrB p	C T G A A A G G G C T G G A T G C G G T G C G T
005 <i>P. mirabilis</i> persister gyrB w-t	C T G A A A G G G C T G G A T G C G G T G C G T
028b <i>K. pneumoniae</i> gyB	C T G A A A G G G C T G G A T G C G G T G C G T
028b <i>K. pneumoniae</i> gyB persister	C T G A A A G G G C T G G A T G C G G T G C G T
005 <i>P. mirabilis</i> persister gyrB p	T A T A T C G G C G A C A C G G A T G A C G G C
005 <i>P. mirabilis</i> persister gyrB w-t	T A T A T C G G C G A C A C G G A T G A C G G C
028b <i>K. pneumoniae</i> gyB	T A T A T C G G C G A C A C G G A T G A C G G C
028b <i>K. pneumoniae</i> gyB persister	T A T A T C G G C G A C A C G G A T G A C G G C
005 <i>P. mirabilis</i> persister gyrB p	A T G G T A T T C G A G G T G G T A G A T A A C
005 <i>P. mirabilis</i> persister gyrB w-t	A T G G T A T T C G A G G T G G T A G A T A A C
028b <i>K. pneumoniae</i> gyB	A T G G T A T T C G A G G T T G T G G A T A A C
028b <i>K. pneumoniae</i> gyB persister	A T G G T A T T C G A G G T T G T G G A T A A C
005 <i>P. mirabilis</i> persister gyrB p	C T C G C G G G T C A C T G T A A A G A A A T T
005 <i>P. mirabilis</i> persister gyrB w-t	C T C G C G G G T C A C T G T A A A G A A A T T
028b <i>K. pneumoniae</i> gyB	C T C G C G G G T T A C T G C A A A G A T A T C
028b <i>K. pneumoniae</i> gyB persister	C T C G C G G G T T A C T G C A A A G A T A T C
005 <i>P. mirabilis</i> persister gyrB p	G C C G A T A A C T C T G T C T C T G T A C A G
005 <i>P. mirabilis</i> persister gyrB w-t	G C C G A T A A C T C T G T C T C T G T A C A G
028b <i>K. pneumoniae</i> gyB	A G C G A T A A C T C C G T C T C C G T C C A G
028b <i>K. pneumoniae</i> gyB persister	A G C G A T A A C T C C G T C T C C G T C C A G
005 <i>P. mirabilis</i> persister gyrB p	A T T C C G A C C G G T A T T C A C C C G G A A
005 <i>P. mirabilis</i> persister gyrB w-t	A T T C C G A C C G G T A T T C A C C C G G A A
028b <i>K. pneumoniae</i> gyB	A T C C C G A C C G G C A T T C A C C C G G A A
028b <i>K. pneumoniae</i> gyB persister	A T C C C G A C C G G C A T T C A C C C G G A A
005 <i>P. mirabilis</i> persister gyrB p	G C G G A A G T G A T C A T G A C C G T T C T G
005 <i>P. mirabilis</i> persister gyrB w-t	G C G G A A G T G A T C A T G A C C G T T C T G
028b <i>K. pneumoniae</i> gyB	G C G G A A G T T A T C A T G A C C G T C C T G
028b <i>K. pneumoniae</i> gyB persister	G C G G A A G T T A T C A T G A C C G T C C T G
005 <i>P. mirabilis</i> persister gyrB p	T T T G A C G A T A A C T C C T A T A A A G T G
005 <i>P. mirabilis</i> persister gyrB w-t	T T T G A C G A T A A C T C C T A T A A A G T G
028b <i>K. pneumoniae</i> gyB	T T C G A T G A T A A C T C C T A T A A A G T T
028b <i>K. pneumoniae</i> gyB persister	T T C G A T G A T A A C T C C T A T A A A G T T
005 <i>P. mirabilis</i> persister gyrB p	G G C G T T G G T G T T T C G G T A G T A A A C
005 <i>P. mirabilis</i> persister gyrB w-t	G G C G T T G G T G T T T C G G T A G T A A A C
028b <i>K. pneumoniae</i> gyB	G G C G T A G G C G T C T C G G T G G T T A A C
028b <i>K. pneumoniae</i> gyB persister	G G C G T A G G C G T C T C G G T G G T T A A C
005 <i>P. mirabilis</i> persister gyrB p	C T G G A G C T G G T T A T C C A G C G C G A G
005 <i>P. mirabilis</i> persister gyrB w-t	C T G G A G C T G G T T A T C C A G C G C G A G
028b <i>K. pneumoniae</i> gyB	C T G G A G C T G G T T A T C C A G C G C G A T
028b <i>K. pneumoniae</i> gyB persister	C T G G A G C T G G T T A T C C A G C G C G A T

**Figure C 2** Multiple Sequence Alignment of gyrB protein-coding sequences for persister cells and wild-type *K. pneumoniae* and *P. mirabilis*

luxS multiple sequence alignment for 5 Gram-negative strains

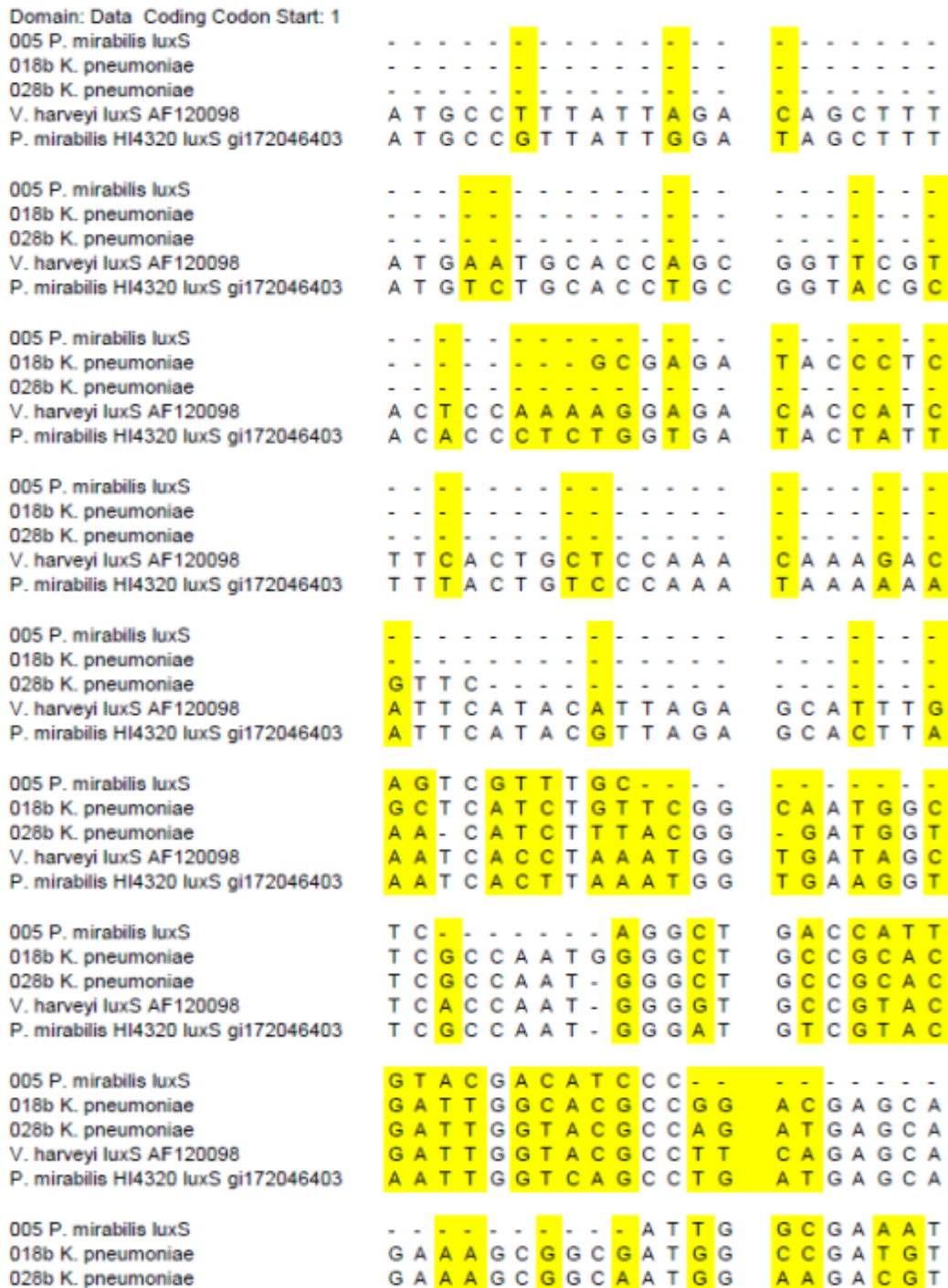
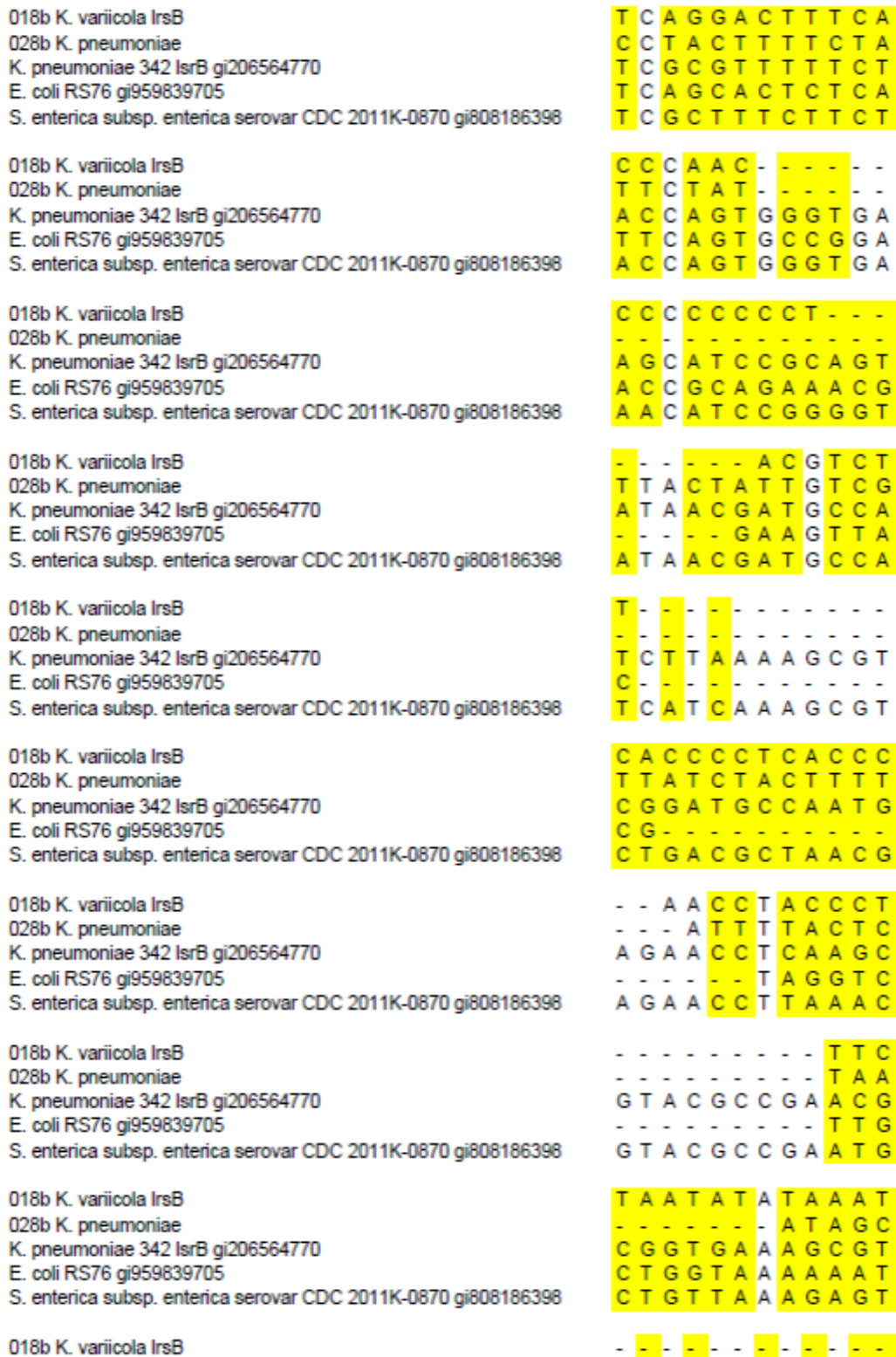
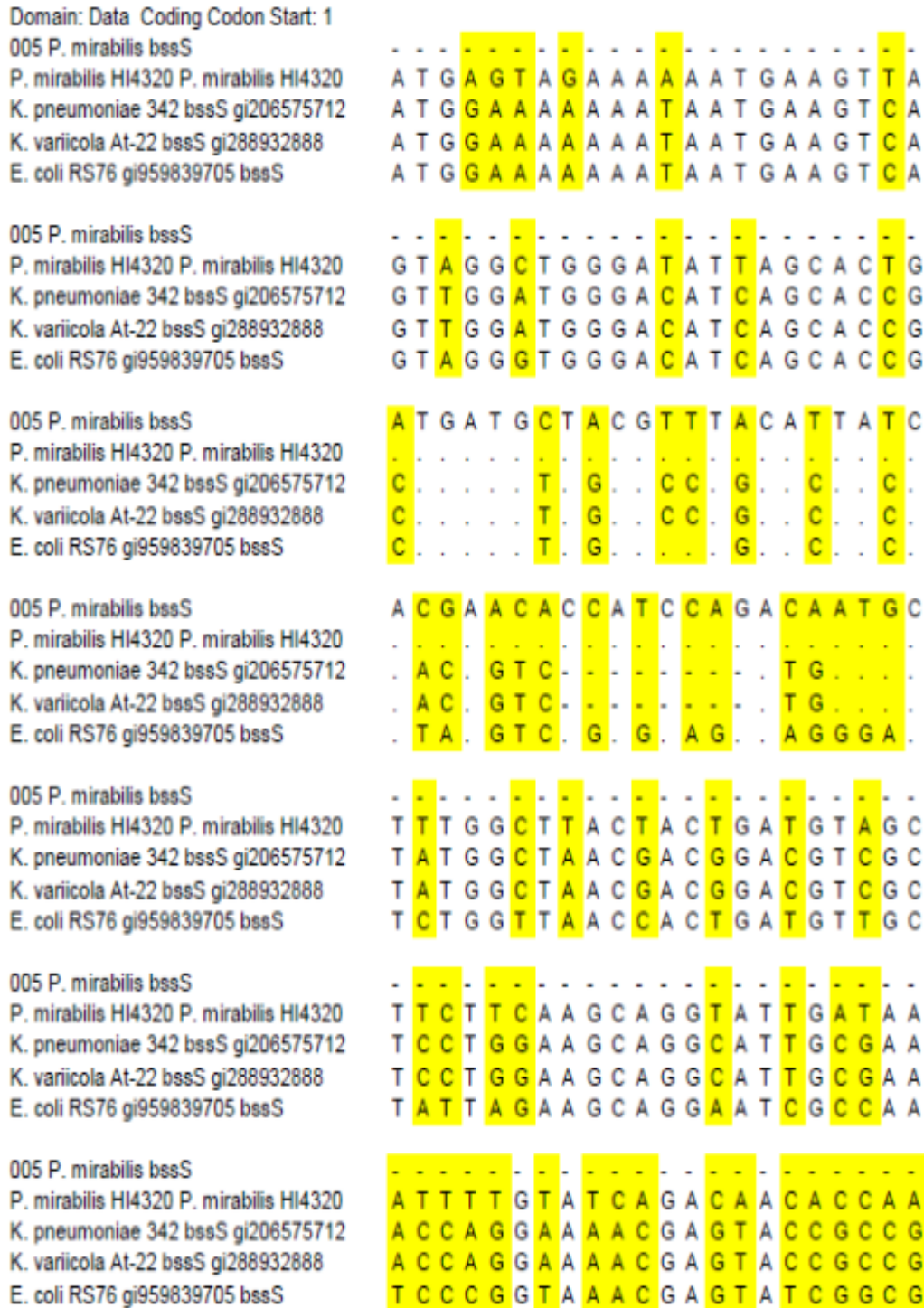


Figure C 3 LuxS multiple sequence alignment. Variable regions highlighted in Yellow



**Figure C 4** LsrB multiple sequence alignment in 5 Gram–negative strains. Variable regions highlighted in yellow



**Figure C 5** BssS multiple sequence alignment in 5 Gram–negative strains. Conserved regions highlighted in yellow

bssR-bssS multiple sequence alignment in 5 Gram-negative strains

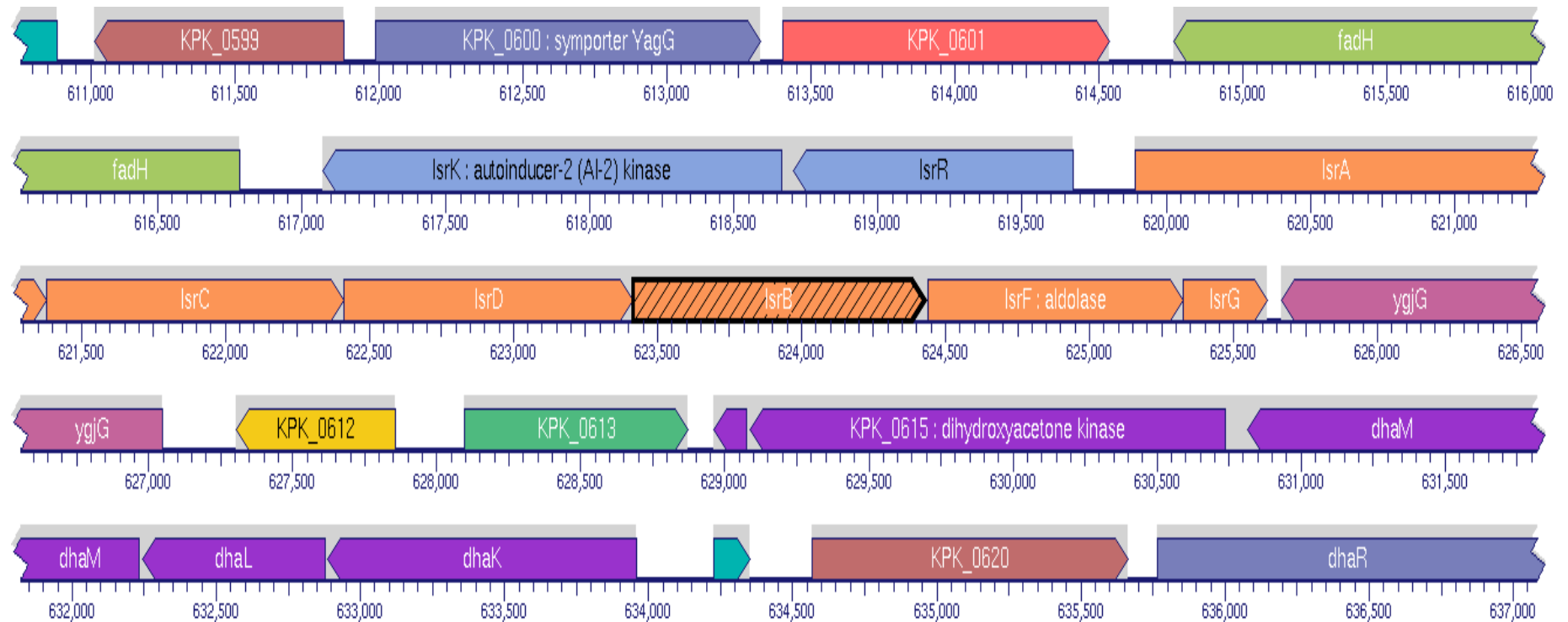
yellow area showing conserved gene sites.

Domain: Data Coding Codon Start: 1

005 <i>P. mirabilis</i> bssS	- - - - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	- - - - -
<i>K. pneumoniae</i> 342 bssR gi206575712	- - - - -
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	A T G T T C G T T G A C A G A C A
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	- - - - -
005 <i>P. mirabilis</i> bssS	- T A A A C G - - - - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	- - - - -
<i>K. pneumoniae</i> 342 bssR gi206575712	G T G A T T G G C G A C A A T T T
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	T T G A T C G A C G C A C G C G T
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	G T A A G C G G T G A A T G T G C
005 <i>P. mirabilis</i> bssS	- - - - - C A G A - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	A T G A A G T T A T T C A A A C A
<i>K. pneumoniae</i> 342 bssR gi206575712	G T C A A T C C T G T C A G A C A
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	A C T G A G G A A G G C A A A - A
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	G T C A A A G T T G T C A G A C A
005 <i>P. mirabilis</i> bssS	- - - - - - - - - - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	T T A G C A C T G T A - - - - - G
<i>K. pneumoniae</i> 342 bssR gi206575712	A C A G C G A T G T A C A G C G G
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	G C A A T C A T C T A C G A G A T
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	A C G G C A A T A A A G G T C G G
005 <i>P. mirabilis</i> bssS	- - - - - G A T G A - - T G
<i>P. mirabilis</i> HI4320 bssS gi197283915	- - - - - G A T G A - - T G
<i>K. pneumoniae</i> 342 bssR gi206575712	A - - - - G G C A T A C T G
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	A A C T G C A C G A T A A A T C G
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	A - - - - A A C A A A C G G
005 <i>P. mirabilis</i> bssS	- - - - - - - - - - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	- - - - - - - - - - -
<i>K. pneumoniae</i> 342 bssR gi206575712	C G C C A G G C G A A G G G C C T
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	A T C A G G A A G A G T G G A G T
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	G G C A C G G C G C A G C G C G T
005 <i>P. mirabilis</i> bssS	- - - - - - - - - - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	- - - - - - - - - - -
<i>K. pneumoniae</i> 342 bssR gi206575712	A T T C T G C A G G C G C G G G G
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	C A T T A G C G A C A G C C G - -
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	C A G T C C C T G C C G T A A A G
005 <i>P. mirabilis</i> bssS	C C A T C C A G A C A A T G C - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	C C A T C C A G A C A A T G C A G
<i>K. pneumoniae</i> 342 bssR gi206575712	A A G C T C A A A C A A G T T A T
<i>E. coli</i> str. K-12 substr. MC4100 bssR gi557270520	- - - - - - C A G T A T G T
<i>S. enterica</i> subsp. <i>enterica</i> serovar P-stx-12	C A G T T C A A A A A A A T T G T
005 <i>P. mirabilis</i> bssS	- - - - - - - - - - -
<i>P. mirabilis</i> HI4320 bssS gi197283915	G G C T T A C T A C T G - - - -
<i>K. pneumoniae</i> 342 bssR gi206575712	C G C T G A C T G A C A T G T A G

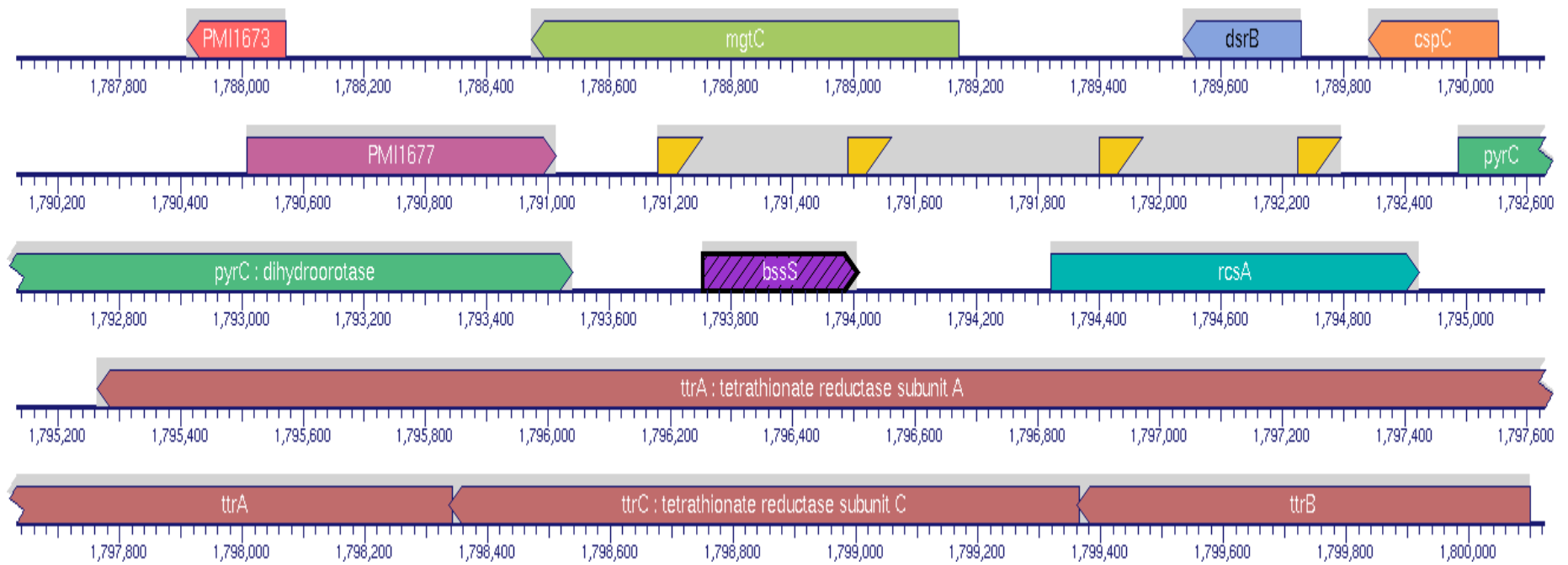
Figure C 6 BssR–BssS multiple sequence alignment in 5 Gram–negative strains. Variable regions highlighted in yellow.

## 7.1.5 Appendix D



**Figure D 1** *lsrACDBFG* operon.

BioCyc Genome Browser map showing the location of the *lsrACDBFG* operon (in orange) in the chromosome of *K. pneumoniae* 342. *LsrR* (repressor) *LsrK* (AI-2 kinase) genes are transcribed in opposite direction to the main operon (image available from <http://biocyc.org/tmp/JB880.gif>).



**Figure D 2** *bssS* Transcriptional unit

BioCyc Genome Browser map showing the chromosomal location of the *bssS* transcriptional unit (in purple) in *P. mirabilis* HI4320 (image available from <http://biocyc.com/tmp/JB967.gif>).

## 7.1.6 Appendix E

### 1. Ethical approval from the University of Westminster



**PRIVATE AND CONFIDENTIAL**  
George Gyamfi-Brobby  
School of Life Sciences

17 February 2013

Dear George

**Ethics App. No. 11-12-41**  
**George Gyamfi-Brobby: School of Life Sciences**  
**Mode: MPhil/PhD**  
**Supervisor: Patrick Kimmitt**

**Title: Diabetic foot ulcers in Ghana**

The Committee considered the proposal and response to conditions on 31 January 2013, the application was approved and the following conditions were set (which may be signed off if completed appropriately by your Supervisor, Patrick Kimmitt):

Please remove from the Consent Form; 'I have read and understood'.  
Participant Information Sheet; simplify the information; the second paragraph is sufficient in explaining the research.  
Consent Form; please do not collect data through this form, no data should be collected until after consent has been received.

If your protocol changes significantly in the meantime, please contact me immediately, in case of further ethical requirements.

Yours sincerely  
Huzma Kelly  
Secretary, Research Ethics Sub Committee

cc. Dr. John Colwell (Chair, Research Ethics sub Committee)  
Patrick Kimmitt (Supervisor)  
Mike Fisher (Research Degrees Manager)

Academic Services Department  
101 New Cavendish Street  
Cavendish House, University of Westminster  
London, W1W 6XH  
T: +44 (0) 20 7911 5051  
E: H.Kelly01@westminster.ac.uk  
westminster.ac.uk/research



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Registration number 977818 England  
Registered office 309 Regent Street London W1B 2UW



## 2. Letter of Collaboration and external supervision



KWAME NKURUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY  
COLLEGE OF HEALTH SCIENCES

**SCHOOL OF MEDICAL SCIENCES**  
**DEPARTMENT OF CLINICAL MICROBIOLOGY**

July 11, 2012

Chair,  
University of Westminster Research Committee  
University of Westminster  
W1W 6UW  
London  
UK

Dear Sir/Madam,

**EXTERNAL SUPERVISION OF PHD CANDIDATE GEORGE GYAMFI-BROBBEY**

I write to confirm that I have accepted to provide the needed support and advice concerning the PhD project of George Gyamfi-Brobbeey, at the Komfo Anokye Teaching Hospital, Kumasi, Ghana.

I am currently the Head of Department of Medical Microbiology, School of Medical Sciences, KNUST, and Honorary Consultant Microbiologist, Komfo Anokye Teaching Hospital, Kumasi.

Please find attached my CV.

Yours faithfully,

Dr. Enoch H. Frimpong  
Head/Professor in Medical Microbiology

### 3. Ethical Approval and Project registration KATH, Kumasi Ghana

**KOMFO ANOKYE  
TEACHING HOSPITAL**



P. O. Box 1934  
KUMASI - GHANA  
Tel: +233-51-22301-4  
Fax: +233-51-24654/24621  
Website: www.kathsp.org

Our Ref. No.: DD/KATH/103.....

Your Ref. No.:.....

Diagnostics Directorate.

Kumasi – Ashanti.

Ghana – West Africa.

20<sup>th</sup> January 2013.

The Research Ethics Sub-Committee.  
Academic Services Department,  
101 New Cavendish Street, Cavendish House,  
University of Westminster,  
London, W1W 6XH

Dear Sir,

**LETTER OF COLLABORATION AND ETHICAL PERMISSION (RE: DIABETIC FOOT ULCERS IN GHANA)**

With respect to the application made by George Gyamfi-Brobbe, PhD student of your institution, to collect clinical samples from diabetic foot ulcer patients attending the Komfo Anokye Teaching Hospital, Kumasi, Ghana, I wish to inform you that his project has been duly registered by the Research & Development Unit and permitted to take place at the premises of the Komfo Anokye Teaching Hospital, Kumasi, Ghana.

During his time at KATH, George will be given all the needed support including facilities that will support the collection and processing of his clinical samples in Ghana.

Thank you and looking forward to strengthening this collaboration in the future.

Yours sincerely,

Dr. Augustina Badu-Peprah (Mrs)  
Head, Diagnostics Directorate,  
KATH, Kumasi – Ghana.

4. Certificate of project Registration, KATH, Kumasi, Ghana



KOMFO ANOKYE TEACHING HOSPITAL  
RESEARCH AND DEVELOPMENT UNIT (R & D)  
**CERTIFICATE OF REGISTRATION**

REG. NO. RD/CR12/035

This is to certify that

Prof./Dr./Mrs./Mr./Ms. Gyampfi Brobbey George  
has registered his/her proposed study titled Diabetic Foot Infections  
.....  
.....with the Research and Development Unit.

Date 16th March, 2012

Name of issuing officer

Dr. Daniel Ansong (Dep. Director, R&D)

Signature

<sup>a</sup>Must tally with registration number on the registration form

## 7.1.7 PARTICIPANT INFORMATION SHEET

### **Project Title: Diabetic foot Ulcers in Ghana**

You are kindly invited to take part in a research project, designed by the University of Westminster (UoW), London, UK in collaboration with the Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana. Mr. George Gyamfi–Brobbe, the project student and the study team will provide you with all the information concerning the project and your participation. Do not hesitate to contact the study team if there is anything you do not understand. You can confirm your participation by signing or thumb–printing the consent form below.

#### **What is the purpose of the study?**

Chronicity of diabetic foot ulcers (DFU) with attendant amputations is largely believed to be due to mutual attachment and interactions among colonising bacteria. This study therefore hypothesises that the pre–treatment of bacteria in a biofilm with sugars and lectins blocking host lectins and sugars respectively can inhibit bacterial attachment and interaction and subsequent disruption of biofilms in diabetic foot ulcers. To fully understand the microbiology and polymicrobial nature of DFUs, this study will investigate the mechanisms underlying biofilm formation in diabetic foot infections and consider the possible development of strategies to inhibit biofilm development through the use of glycan–lectin analogues and quorum quenching.

The study involves collaboration with the Microbiology departments at the Kwame Nkrumah University of Science and Technology and the University of Ghana Medical School, Ghana.

The study aims to establish the prevalence and incidence rates of diabetic foot infections among diabetics in Ghana and provide a data on the microbiology of the infections. Data from the study will be used to inform health policies on diabetes management in Ghana; provide education and serve as foundation for future researches on diabetes in Ghana and Africa as a whole.

#### **What will you be asked to do?**

Participation in this study is voluntary. Wound specimens will be collected from you as part of your routine clinic appointment. A sterile cotton swap will be introduced into your wound to collect the sample. This will be done by an experienced staff of the clinic/ward in the safest and most non–invasive way possible. You have every right to withdraw from the exercise if you are not comfortable or unwell. The sample that you provide will be processed immediately for the detection of the bacteria that colonise the wound.

#### **Risks and Discomfort**

It is very unlikely that there will be any side effects for taking part in the study. You may experience slight discomfort when wound swaps are being taken. However, this will be done in the safest and most non–invasive way as possible.

#### **Why have I been asked to participate?**

You have been asked to participate because of your present condition and history of being diabetic with foot ulcer/infection.

#### **Do I have to take part?**

Participation is voluntary. However, if you decide to take part, a copy of this information sheet will be given to you to keep. You are free to withdraw at any time without giving reasons and can request the removal of your sample from the study. Moreover, your decision to withdraw will not affect your eligibility for any future research.

### **What do I have to do?**

In order to be recruited for the study, you are kindly requested to answer some few simple questions below. You can then confirm your participation by signing the consent form below.

### **Confidentiality**

This is a student research project which may be published. In the course of the project and in the event of subsequent publication, your participation, name and any other personal details will be kept highly confidential. Your sample will be given a specific research number and anonymised. Access to identifiable data will be held in Ghana **only** by your respective GPs who have access to your information. Dr Patrick Kimmitt, Dr Pamela Greenwell and George Gyamfi–Brobbe will **only** handle anonymised samples with no bearing to your identity.

### **Expenses and Payments**

Participation is entirely voluntary and as such there will be no payments for your participation in the study.

### **Contact for further information**

If you have any problem or query about any aspect of the study at any time, please do not hesitate to contact the researchers on their contacts given below:

PhD student:	George Gyamfi–Brobbe <a href="mailto:ggyamfb@gmail.com">ggyamfb@gmail.com</a> +44 7852 946933/ +233 20 7967849
Director of studies:	Dr Patrick Kimmitt <a href="mailto:p.kimmitt@westminster.ac.uk">p.kimmitt@westminster.ac.uk</a> +44 20 7911 5000 EXT 3668/ 64135
Second Supervisor:	Dr Pamela Greenwell <a href="mailto:greenwp@westminster.ac.uk">greenwp@westminster.ac.uk</a> 020 7911 5000 EXT 64147
External Collaborator	Prof Enoch H. Frimpong <a href="mailto:ehfrimps@yahoo.co.uk">ehfrimps@yahoo.co.uk</a> +233 20 8124866

### 7.1.8 Participant's Questionnaire

#### Question we need to ask you:

Which part of Ghana do you come from? .....

How long have you been diagnosed as being diabetic? .....

Have you had an ulcer/wound on your foot before? Yes/ No

If yes, when did it first appear? ..... Size/Grade.....

Have you been attending the diabetic clinic regularly? Yes/ No

If yes, for how long? .....

If no, why? .....

Do you smoke? Yes/No

Do you live alone? Yes/No

Do you often wear, a) flip-flops, b) shoes with socks or c) walk bare foot?

Do you have hypoglycaemia (low sugar level) attacks? Yes/ No

If yes, how often? .....

Do you have hyperglycaemia (high sugar level) attacks? Yes/ No

If yes, how often? .....

### 7.1.9 PARTICIPANT CONSENT FORM

Project Title: Diabetic foot ulcers in Ghana

Statement by subject

I confirm that I have been provided with sufficient information about this project.

I understand that I have volunteered to take part and can withdraw at any time, without being disadvantaged and without giving my reasons.

I am satisfied that the results will be stored securely.

I know that the results will not be linked to me and will be kept confidential.

I am aware of any possible risks and discomfort.

I agree to inform the researcher immediately if I feel uncomfortable or experience any discomfort.

I have had the chance to ask questions.

I know that I will not receive any money for taking part.

I agree to take part in this project.

Participant Details

Name: .....

Age: .....

Sex: Male/Female

Signature/Thumbprint: .....

Date of Study: .....

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