The role of catecholamine stress hormones and inotropes in the promotion of bacterial growth, virulence and biofilm formation

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Abstract

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Bacteria in most environments exist and grow in association with surfaces, leading to formation of biofilms. In the medical context, biofilms are particularly significant for human health because of their high resistance to antimicrobial and immune system attack. In the health care setting biofilms associated with indwelling devices such as intravenous catheters and endotracheal tubes is a major clinical problem. It has been shown in previous reports that catecholamine stress hormones such as epinephrine, norepinephrine and structurally similar inotrope drugs used to treat heart and kidney problems in seriously-ill patients, are able to promote growth and virulence of certain bacteria. In this thesis, the role of catecholamine inotropes and stress hormones as an environmental factor for the induction of biofilm formation by infectious bacteria relevant to medical devices is considered. *In vitro* phenotypic characterisation was investigated by mainly using microbiological techniques, microscopy and proteomic analysis.

The first section of this thesis shows how clinically attainable levels of catecholamine inotropes stimulated *Pseudomonas aeruginosa* growth and biofilm formation. The mechanism by which growth stimulation occurs was found to be via delivery of iron from the serum Fe-binding transferrin. *P. aeruginosa* growth, biofilm formation and motility were all significantly enhanced by catecholamine inotropes ($P<0.05$). Inotropes may be a risk factor for ventilator associated pneumonia as they stimulated biofilm formation on endotracheal tubing. In the second section which focuses on *Escherichia coli* and *Salmonella Typhimurium,* it was found that catecholamine stress hormone effect on growth, motility and biofilm formation was independent of the putative QseC and QseE catecholamine sensing receptors. In the third section, factors related to catecholamines inotropes *Staphylococcus epidermidis* biofilm stimulation were considered.

Collectively, these findings show that levels of catecholamine inotropes found within critically ill patients can promote bacterial biofilm formation, and so contribute to bacterial pathogenesis within the hospital setting.

Dedicated to my beloved late father (May God's mercy be upon him) & my beloved mother

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Abbreviations

AE	Attaching and Effacing
AHL	acyl-homoserine lactone
AI	Auto Inducer
Bap	Biofilm associated proteins
BSA	Bovine Serum Albumin
CDC/NHSN	Centers for Disease Control and prevention/ National Healthcare Safety Network
CFU	Colony Forming Unit
CoNS	Coagulase Negative Staphylococci
cpm	Counts per minute
CSLM	Confocal scanning electron microscopy
CV	Crystal Violet
DMEM	Dulbecco Modified Eagle's Medium
DNA	Deoxy Ribose Nucliec Acid
Dop	Dopamine
EB	Envelope buffer
e-DNA	extracellular DNA
EDTA	Ethylene Diamine Tetra Aceticacid
EHEC	Entero Haemarrhagic *E.coli*
ENS	Enteric Nervous System
Epi/Ad	Epinephrine/Adrenaline
EPS	Exopolymeric substances
ET	Endotracheal Tube
Fur	Ferric uptake regulatory system
GFP	Green Fluorescent Protein
HC	Haemorrhagic Colitis
HPA	Hypothalamic Aituitary Adrenal axis
HUS	Hemolytic-Uremic Syndrome
ICU	Intensive Care Unit
IV	Intra Venous

Kan-	kanamycin
LB	Luria Bertani broth
LEE	Locus of Enterocyte Effacement
MSCRAMM-	Microbial surface components recognizing adhesive matrix molecules
Nal^R	Nalidixic acid resistant
NE	Norepinephrine /Noradrenaline
PBS	Phosphate Buffered Saline
PIA	Polysaccharide Intercellular Adhesin
PSMs	Phenol Soluble Modulins
QS/QSe	Quorum Sensing
SB	Sample loading buffer
SAPI	Standard American Petrolium Institute
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
SPIs	Salmonella Pathogenicity Islands
Stx	Shiga toxins
TEM	Transmission Electron Microscopy
TEMED	N,N,N'N'-Tetramethylethylenediamine
Tir	Translocated intimin receptor
Tris	Tris (hydroxymethyl) aminomethane
TSB	Tryptone Soy Broth
VAP	Ventilator Associated Pneumonia
WT-	wild type
Δ mutant	deletion mutant

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Chapter One

Introduction

1. Introduction

1.1. Biofilms

1.1.1 What is a biofilm?

Bacteria in the majority of environments are not found as unicellular planktonic forms or as pure cultures studied in the laboratories, but instead exist as multicellular sessile forms called biofilms (Costerton *et al*., 1995, Davey and O'Toole, 2000). Biofilms have been defined in many ways by many researchers depending on the environments in which they form. A more generalised definition is 'a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription' (Donlan and Costerton, 2002).

In the 17th century, Antony van Leeuwenhoek was the first person to observe and discover biofilms on tooth surfaces using his simple microscopes. Claude Zobell in 1934 discovered the preference of marine bacteria to attach to surfaces. Based on observations of dental plaque and attached communities in mountain streams, Costerton *et al*. in 1978 presented a theory of biofilm formation that explained the mechanisms by which microorganisms attach to biotic and abiotic materials, and suggested the relevance of these multicellular communities (Costerton *et al*., 1978, Donlan, 2002).

Biofilms form in every environment as long as a surface for attachment, nutrients and water are available. Biofilms are ubiquitous in nature and are found in many industrial and medical settings (Dirk De Beer and Stoodley, 2006). Major features characterising biofilms are the production of exopolymeric substances (EPS), antimicrobial resistance, structural heterogeneity, community interactions and genetic diversity (Donlan, 2002).

1.1.2 Formation of biofilms

Biofilm formation can occur by at least three mechanisms (Stoodley *et al*., 2002); one is by the redistribution of attached cells by pili mediated twitching motility, a second mechanism is from binary division of attached cells leading to cell clusters similar to colony formation in agar plates. The third mechanism is the recruitment of cells from the bulk fluid to the developing biofilm. The occurrence of each of these mechanisms will depend on the organism involved, characteristics of the surface being colonized, and the physical and chemical conditions of the environment.

The biofilm life cycle is divided into three main stages: attachment, growth and detachment. A five stage universal biofilm development cycle has been proposed with common characteristics independent of the phenotype of the organisms. These five stages have been proposed largely on the basis of proteomic studies in *Pseudomonas aeruginosa* (Figure.1.1) (Sauer *et al*., 2002).

A bacterial biofilm begins to form when individual bacteria attach to a surface. This initial attachment is also called adsorption and is influenced by electrical charges carried on the bacteria, by Van der Waals forces and electrostatic attraction. This transient adherence can result in a stable irreversible attachment or return to the planktonic mode (Palmer and White, 1997). The initial colonisation event is governed by both environmental factors and genetics (Hall-Stoodley *et al*., 2004). In motile bacteria localization to a surface occurs by flagellar mediated motility, whereas, non-motile bacteria increase the expression of adhesins (Lemon *et al*., 2008). Cell surface hydrophobicity, presence of fimbriae and flagella, and production of EPS all influence the rate and extent of attachment of microbial cells (Donlan, 2002). After initial attachment, the bacterial cells grow, divide and spread on the surface as a monolayer to

form microcolonies. During microcolony formation, cells undergo developmental changes, which give rise to the exopolymeric substances matrix and the complex architecture of the mature biofilm with its extensive cellular differentiation (Finkel *et al*., 2003, Lemon *et al*., 2008). Single cells detach from the mature biofilm due to external factors such as shear stress and internal processes such as enzymatic degradation or surface binding proteins or EPS release (Hall-Stoodley *et al*., 2004). The dispersed cells then re-enter the planktonic mode and repeat the biofilm cycle, when conditions are favourable.

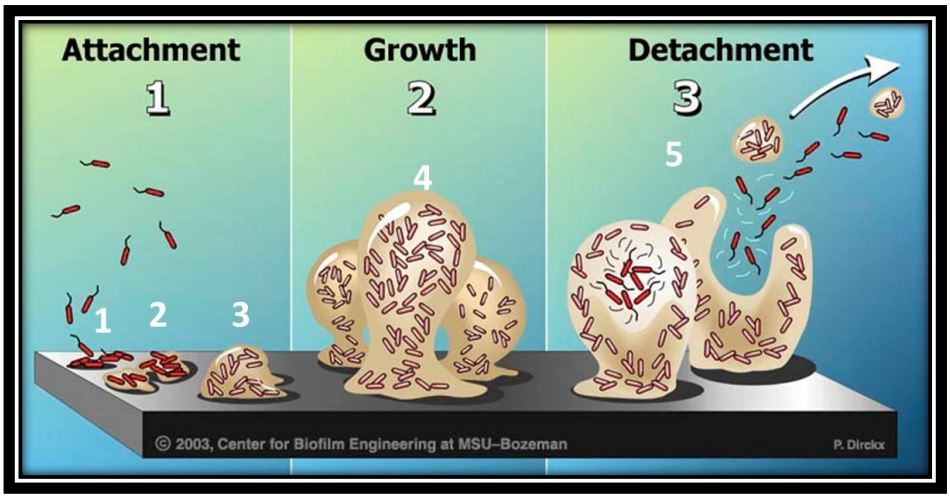


Figure.1.1: The biofilm development stages (taken from Centre for biofilm engineering, Montana University, image archives, with permission)

1: Individual cells colonise the surface. 2: EPS is produced and attachment becomes irreversible. 3 & 4: biofilm architecture develops and matures. 5: single cells are released from the biofilm in the detachment stage

1.1.3 Biofilm Structure

In nature biofilms are usually not monospecies as studied in the laboratory, rather they are composed of a mixture of many species of bacteria, as well as fungi, algae, yeasts, protozoa and other microorganisms, debris and corrosion products (Palmer and White

1997, Stoodley *et al*., 2002). However, in the medical and industrial settings biofilms can exist as pure cultures. Structural similarities have been observed between single species *in vitro* biofilms and mixed species biofilms formed in nature (Davey and O'Toole 2000).

Biofilm structure is the spatial arrangement of bacterial cell clusters, extra polymeric substances (EPS) and particulates. The physical properties of biofilms are largely determined by the EPS, while the physiological properties are determined by the bacterial cells (De Beer and Stoodley, 2006). Various views on the structure have been proposed and three conceptual models have been derived from biofilms on the basis of substrate concentration and flow conditions (Wimpenny *et al*., 2000) i. simple planar homogeneous structures based on dental plaque observations ii. biofilms consisting of stacks of bacterial colonies attached to a substratum, embedded in extracellular polymeric substances (EPS) and appearing as columns surrounded by the bulk liquid (also known as heterogeneous mosaics), these have been described in water distribution systems (Keevil and Worker, 1992) iii. Three dimensional complex architecture with the widely recognised mushroom shaped structures and water channels revealed by confocal scanning laser microscopy (CSLM) and fluorescent particles (deBeer *et al*., 1994). Many mathematical models have also been proposed to describe the structure and function of biofilms (Characklis, 1990, Rittmann and Manem, 1992).

1.1.3.1 Biofilm Matrix

The matrix which forms the immediate environment of cells is the main structural component of biofilms. The matrix is hydrated extracellular material usually self-produced by the organism, in which the biofilm cells are embedded. EPS was once

denoted as ‘extracellular polysaccharides’, later was named as ‘extracellular polymeric substances’ due to the presence of other biopolymers (Flemming *et al*., 2007). The matrix is composed of cells, water and extracellular polymers. In most biofilms, the biofilm cells account for less than 10% whereas, the matrix can account for over 90% of the dry biomass (Allison, 2003, Flemming and Wingender, 2010). The biofilm matrix is a dynamic environment, as it constantly changes according to the surrounding environment (Sutherland, 2001).

The major component of the biofilm matrix is water which provides a hydrated environment and protects the biofilms from water fluctuations in the environment. Matrix components are diverse and vary dependent on the microbial species and environmental conditions. The EPS comprise of polysaccharides, a wide variety of proteins, glycoproteins, glycolipids, extracellular DNA (e-DNA) (Sutherland, 2001, Flemming, 2007, Pamp *et al*., 2007).

Polysaccharides are a major fraction of the EPS matrix and vary among bacteria (summarised in Table 1.1). The exopolysaccharide composition and its physical properties are determined by the type of monomer units and the glycosidic linkages (Flemming and Wingender, 2010). The matrix also contains proteins and can sometimes exceed the polysaccharide content ratio in environmental biofilms (Flemming and Wingender, 2010). Cellular appendages such as flagella, fimbriae, pili, curli are mainly composed of proteins and surface associated proteins such as Bap (biofilm associated proteins) in *S. aureus*, *Salmonella*, all function as important parts of the EPS matrix. These proteins are involved in the promotion of cell aggregation, surface adhesion and biofilm formation (Pamp *et al*., 2007). Extracellular DNA (e-DNA) also acts as a matrix component in bacteria such as *P. aeruginosa*, *Streptococcus*

spp., *Bacillus subtilis*, *Neisseria gonorrhoeae* and are mainly found in waste water biofilms (Pamp *et al*., 2007).

The regulatory mechanism involving proteins with GGDEF and or EAL domain controls the expression of polysaccharides and proteins. These proteins by controlling the level of a second messenger, the cyclic dinucleotide c-di-GMP, regulate matrix production and adhesiveness of bacteria (D'Argenio and Miller, 2004, Pamp *et al*., 2007). Extracellular DNA is regulated via quorum sensing (Pamp *et al*., 2007).

Table.1.1 Different exopolysaccharides produced by bacteria (Adapted from Pamp *et al*., 2007)

Exopolysaccharide	Organisms
Cellulose	*E. coli*, *Salmonella spp*., *Pseudomonas fluorescence*, *Agrobacterium tumefaciens*, *Glucanobacter xylinus* etc
PNAG/ PIA	*S. aureus*, *S. epidermidis*
Alginate Pel, Psl	*P. aeruginosa*
VPS	*Vibrio cholerae*
Colanic acid	*E. coli* and many Enterobacteria

Legend: Pel- pellicle VPS- Vibrio polysaccharide, PNAG poly-N-acetylglucosamine)/ PIA (polysaccharide intercellular adhesin)

The chemical and physical nature of the biofilm matrix protects organisms against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations,

ultraviolet radiation, many protozoan grazers and host immune defences. The presence of EPS has been linked with many processes and properties integral to biofilm behaviour, such as attachment, detachment, mechanical strength, antibiotic resistance, biofilm architecture and exo-enzymatic degradation activity (Flemming & Wingender, 2010). Davies *et al*. (1998) has shown that biofilms maintain their structural heterogeneity by releasing EPS-degrading enzymes, the regulation of which involves quorum sensing. In addition, the biofilm matrix acts as a nutrient source and an external digestive system by keeping extracellular enzymes close to the biofilm cells, enabling them to metabolize dissolved, colloidal and solid biopolymers (Flemming and Wingender, 2010). Xavier and Foster (2007) have demonstrated that EPS pushes younger cells up and out into better nutrient oxygen conditions, suffocating neighbouring non EPS producers, thereby benefitting the principal biofilm organisms.

EPS production is affected by nutrient concentration and physical parameters and thus affects the nature of laboratory biofilms and other bacteria in different environments (Wimpenny and Colasanti, 1997, Stoodley *et al*. 1999a).

1.1.3.2 Biofilm Architecture

Biofilms are not structurally homogeneous monolayers of microbial cells on a surface. Rather, they are described as heterogeneous in both time and space due to constantly changing internal and external processes (Donlan, 2002). According to Tolker-Nielsen and Molin (2000) every microbial biofilm community is unique although some structural attributes can generally be considered universal (Tolker-Nielsen & Molin, 2000). The basic components of bacterial biofilms consist of the cells and the EPS, which can combine to form secondary structures such as microcolonies which may take

on various forms and dimensions such as mushrooms, towers, honey comb-like structures and a base film (Schaudinn *et al*., 2007). The basic structural unit of the biofilm is the microcolony. Microcolonies are separated from each other through 'interstitial voids' or water channels. The structural heterogeneity is important as it modifies the intensity of mass transport to deeper layers of biofilms (Hall-Stoodley *et al*., 2004). Finally, the arrangement of the base film, cell clusters, and the void areas between the clusters, combine to give the overall biofilm architecture. The overall architecture of biofilms is influenced by many physical, environmental and genetic factors including hydrodynamic conditions, concentration of nutrients, bacterial motility and quorum sensing as well as EPS (Hall-Stoodley and Stoodley *et al*., 2002, Hall-Stoodley *et al*., 2004). Mushroom shaped structures and water channels may be seen in the laboratory grown biofilms and natural environment such as streams. However, these structures are not observed in device related biofilms *in vivo* and the *in vitro* structures observed only on certain bacteria (eg:*P.aeruginosa*) are test related artifacts (due to shear stress, flow condition, nutrients etc.) (Personal communication Dr.Bayston).

1.1.4 Environmental impact of biofilms

Biofilms are ubiquitously distributed in natural and industrial systems. They are found in soil and aquatic environments, on tissues of plants, on and within animals as well as in technical systems such as, ship hulls, pipelines, heat exchangers and filters (Costerton *et al*., 1987, 1995). The importance of biofilms in nature, industries and human health is summarised below. Medically important biofilms will be the primary focus, due to its relevant to this study.

1.1.4.1 Natural and industrial biofilms

Bacterial and other microbial biofilms grow in wide variety of environments which includes extreme environments such as thermal springs, ice covers in lakes of Antarctica and deep sea vents (Costerton *et al*., 1987, 1995, Davey and O'Toole, 2000). They play a major role in the production of organic matter, the degradation of many organic compounds and environmental pollutants, and cycling of nutrients (Davey and O'Toole, 2000). Biofilms are a dominant mode of life in streams and are important component of stream food webs and contribute to local energy and nutrient cycling (Fischer and Pusch 2001, Battin *et al*., 2003). In plants, microbial populations are associated with their external tissues and the symbiotic relationship occurring in the rhizosphere between the microorganisms, plant roots, root hairs is a beneficial biofilm association (Davey and O'Toole, 2000). In animals biofilms are found on teeth and oral epithelia and extensively throughout the mucosa of the digestive tract (Dale and Fredericks, 2004).

Biofilms can cause many problems for a wide range of industries and can significantly influence the industrial productivity. Biofilm contamination and fouling occur in almost in every water based industrial process, which includes water treatment and distribution systems, manufacturing systems and the operation of cooling towers etc. However, biofilms are also beneficial, as in bioremediation of hazardous waste sites, microbial leaching, treatment of municipal, industrial wastewater, requires complex microbial community interactions (Costerton, 1987, Davey and O'Toole, 2000).

1.1.4.2 Medical biofilms

Biofilms have found to be involved in a wide variety of microbial infections in the body. According to the National Institutes of Health 2002, over 80% of all human infections are biofilm-related (Davies, 2003). Biofilms have particular significance for human health, because biofilm-associated microorganisms are often less susceptible to antimicrobial agents (Donlan, 2001). Biofilm-associated microorganisms have been shown to be associated with human chronic and persistent infections such as cystic fibrosis, native valve endocarditis, otitis media, and periodontitis and to colonize a wide variety of prosthetic devices or implants (Donlan and Costerton, 2002, Hall-Stoodley *et al*., 2004). Various infections associated with biofilms are summarised in Table 1.2.

From a medical perspective, biofilms can be beneficial, such as the commensal organisms lining a healthy intestine and the female genito-urinary tract, and detrimental in case of persistent and indwelling medical device-related infections (Habash and Reid, 1999). Parsek and Singh (2003) have proposed the following criteria to define medical biofilms a) The infecting organisms are attached to a substratum or surface (b) Direct examination of infected tissue shows bacteria living in microcolonies, encased in an extracellular matrix composed of bacterial and host components. (c) The infection is generally localised and the infection is difficult to eradicate with antibiotics even though the planktonic counterparts are susceptible to antibiotics. Biofilm infections can pose serious clinical challenges for treatment, due to the presence uncultivable species, chronic inflammation, delayed wound healing, antibiotic resistance, and spread of infectious emboli (Bryer, 2008, Hall-Stoodley *et al*., 2004).

Biofilm bacteria can be up to a thousand times more resistant to antimicrobial agents than planktonic bacteria of the same species depending on the organism and type of antimicrobial and experimental system (Parsek and Singh, 2003). Biofilm bacteria are not easily cleared by phagocytosis and antibodies, as the EPS shields antigens and key ligands from the immune system (Costerton *et al*., 1999, Parsek and Singh, 2003). The antimicrobial susceptibility of biofilms may be intrinsic or acquired (Donlan, 2001). Three mechanisms have been proposed for the reason of intrinsic antimicrobial resistance of biofilm bacteria: First, biofilm matrix delays the penetration of the antimicrobial agent by retarding the diffusion due to chemical reaction or limiting the rate of transport. Second, biofilm-associated organisms have reduced growth rates, as slow-growing cells are less susceptible to antimicrobial agents. Third, the environment immediately surrounding the cells within a biofilm may provide conditions that give rise to a protected phenotype. The acquired resistance is due to the exchanges of antimicrobial resistant plasmids to intra- and inter- species by conjugation or horizontal transfer (Costerton *et al*., 1999, Donlan, 2001, Donlan and Costerton, 2002).

Device related biofilms

Usage of indwelling prosthetic devices has become routine in modern medicine. Biofilms-associated infections with indwelling medical devices, particularly plastic made devices can result in significant mortality and morbidity and can increase patient-associated costs due to prolonged hospital stay worldwide (WHO, 2011). Prosthetic devices are used to repair or replace a structure or function of an organ which is damaged or absent as a result of disease, surgery or birth defect (Bayston, 2000). According to the CDC/NHSN (2008) surveillance definition, most nosocomial infections tend to occur at four major body sites: the urinary tract, respiratory tract,

bloodstream, and surgical wound sites; the first three are common sites associated with medical devices. Medical devices are responsible for about 60–70% of hospital-acquired infections, particularly in critically ill patients (Darouiche, 2001, Bryers, 2008).

Medical devices or implants which are commonly associated with infections include: intravascular catheters, prosthetic heart valves, orthopaedic implants, cardiac pacemakers, vascular prostheses, cerebrospinal fluid shunts, urinary catheters, voice prostheses, ocular prostheses (Bryers, 2008) (Table 1.2). In non-surgical indwelling medical devices, such as central venous and urinary catheters, once implanted biofilm colonisation may originate either from the skin microflora, or by migration of the organisms from the skin along the catheter surface or from the catheter hub. As for surgical devices, tissue damage and clot formation associated with surgical implantation contribute to bacterial colonisation (Donlan, 2002, Rodrigues, 2011). Prosthetic devices are made from a range of materials such as silicone elastomer, polyurathanes, polyvinyl chloride, Teflon, titanium stainless steel, ceramics etc (Bayston, 2000). Both material properties and deposition of host factors such as fibronectin, fibrinogen or platelets determine bacterial adhesion to medical devices (Fux *et al*., 2003).

Biofilms on indwelling medical devices may be composed of Gram-positive or Gram-negative bacteria or yeasts. Biofilms may be composed of a single species or multiple species, depending on the device and its duration of use in the patient. Most of the biofilm volume is composed of extracellular polymeric substance which entraps minerals and host plasma components (Donlan, 2001). The source of these organisms may be from the skin of patients, hospital workers, contaminated infusions, hospital water supply or other sources in the immediate environment (Donlan, 2001, Safdar *et al*., 2002)

The initial adhesion begins as soon as a biomaterial is implanted into the body. A conditioning film begins to form by the interaction of the implant and plasma-derived proteins and the surface chemistry of the biomaterial is altered by the adsorption process. Depending on the site of implantation, the conditioning film might consist of fibronectin, vitronectin, laminin, collagen, albumin, immunoglobulins, mucins, haemoglobins, platelets and red blood cell fragments (Bayston, 2000). After the initial attachment of the biofilm bacteria, the organisms which are not cleared by phagocytosis multiply and eventually form plaques; which further develop into microcolonies, produce EPS and form mature biofilms (Bayston, 2000). Depending on the environmental conditions, the biofilms detach and can cause life threatening event called thromboembolism if they become associated with platelets (Bryer, 2008). Mature biofilms formed on medical devices exhibit cell differentiation such as small colony variants (SCV), which is due to nutrient limitation and oxygen: carbon dioxide gradients within the biofilms (Bayston, 2000).

Strategies to prevent biofilm formation range from systemic asespsis-associated approaches controlling any bacterial invasion of sterile sites to inhibition of local biofilm on the actual medical devices (Fux *et al.*, 2003). Effective prophylactic strategies used to reduce the incidence of the medical device associated biofilm infections include device coatings, device immersion, surgical site irrigation, antibiotic loaded cements, and antibiotic lock therapy (Lynch and Robertson, 2008). Devices coating is done with antimicrobials (e.g.: rifampicin, minocycline), hydrogels or metals particularly silver (Bayston and Lambert, 1989, Bayston *et al*., 1997, Bayston, 2000). In the antibiotic catheter lock therapy, the catheter lumen is filled with a concentrated antibiotic solution and then “locked” in a place for a long period in order to prevent bacterial colonisation (Lynch and Robertson, 2008). The increase in resistance to

antibiotics by biofilm organisms have turned researchers to alternative therapy including disinfectants, bacteriophage and bacteriophage lytic enzymes, probiotics, human antimicrobial peptides (defensins, cathelicidins, and histatins) and quorum sensing inhibitors (Donlan, 2008, Rodrigues, 2011).

Table 1.2 Biofilm-related infections (adapted from Costerton *et al*., 1999, Donlan**,** 2002)

Disease or Infection	Common biofilm organism
Persistent Infections	
Dental caries	Acidogenic Gram-positive cocci (e.g., Streptococcus)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of *Haemophilus infuenzae*
Necrotizing fasciitis	Group A streptococci
Biliary tract infection	Enteric bacteria (e.g., *E.coli*)
Osteomyelitis	Various bacterial and fungal species often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
Melioidosis	*Burholderia pseudomallei*
Noscomial and medical device related infections	
ICU pneumonia	Gram-negative rods
Sutures	*S. epidermidis* and *S. aureus*
Contact lens	*P. aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*E. coli* and other Gram-negative rods
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
Intra uterine devices	*Actinomyces israelii* and many others
Endotracheal tubes	A variety of bacteria and fungi
Central venous catheters	*S. epidermidis* and other CoNS
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Vascular grafts	Gram-positive cocci
Orthopaedic devices	*S. aureus* and *S. epidermidis*, *P.acnes*
Penile prostheses	*S. aureus* and *S.epidermidis*
Cerebrospinal fluid shunts	*S.aureus*, *S.epidermidis*, *Propionibacterium acnes* (Bayston, 2000)

1.1.5 Genomics and Proteomics of biofilms

Understanding of the genetic and molecular basis of biofilms will be useful in drug target strategies aimed at the control of biofilm infections. The global gene expression pattern of planktonic and biofilm bacteria can be studied with the combination of microarrays and availability of complete bacterial genome sequences. Proteomics refers to study and analysis of the entire set of proteins expressed under particular set of conditions. Proteins are identified by one-, two-dimensional gel electrophoresis and western blotting. Both approaches suggest that biofilm bacteria differ from planktonic cells but the degree to which they differ will require further application of these techniques (Sauer, 2003, Pratt and Kolter, 1999).

1.1.5.1 Genomic investigation of biofilms

Biofilm-defective mutants have been used to the study the functions of genes required for biofilm development. Two groups of *P.aeruginosa sad* (surface attachment defective) mutants have been isolated that are defective in the capacity for attachment to surfaces (O'Toole and Kolter, 1998a). The first group was defective in flagella and motility and were unable to attach to abiotic surface. The second group was unable to produce type IV pili and spread as a monolayer and form microcolonies and it was proposed from this study that type IV pilus is associated with twitching motility and thus might be the basis of microcolony formation (O'Toole and Kolter, 1998a). Mutation in Type I pili (known as mannose sensitive adhesins), and curli formation in *E.coli* have also shown to lead to defective biofilm formation (Pratt & Kolter, 1998)

Global gene expression analyses have found that different genes of bacteria in biofilms are differentially regulated. The variation in gene expression in biofilm bacteria can range from 1% to 38% of the total genome when compared with planktonic bacteria. Organisms showing such differences include *E. coli, P. aeruginosa, P. putida B. subtilis* and *Shewanella oneidensis* (Sauer, 2003, Southey-Pellig *et al*., 2005) Among the genes that showed increased expression in *E.coli* biofilms were involved in adhesion and auto aggregation, outer membrane proteins (OmpC, OmpF, OmpT, Slp), and proteins involved in lipid A biosynthesis (Schembri *et al*., 2003). In *Bacillus subtilis*, 519 genes were differentially expressed, including those associated with phage-related functions, membrane bioenergetics, glycolysis, and the tricarboxylic acid cycle (Stanley *et al*., 2003). In *P.aeruginosa*, genes up-regulated were involved in motility, attachment, translation, metabolism, transport and regulatory functions, and temperate phage (Whiteley *et al*., 2000). Many other reports have also discussed about genes involved in biofilm formation using transposon mutagenesis and knock-out genes in variety of biofilm bacteria in different settings (Heydorn *et al*., 2002, Beloin and Gigo, 2005, Friedman and Kolter, 2004a, b).

An increasing number of studies have shown that biofilm formation is controlled by complex regulatory pathways (Hall-Stoodley and Stoodley, 2002). In *P.aeruginosa*, the *crc* locus (which codes for catabolite repressor protein and has been shown to regulate carbon metabolism and type IV pili biosynthesis) plays an important role in biofilm development (O'Toole, *et al*., 2000). Other studies have shown regulatory pathways associated with initial attachment, such as the *Cpx* pathway in *E. coli* and *relA* in *Listeria monocytogenes* and *P. fluorescens ClpP* (Otto *et al*., 2002, Taylor *et al*., 2002, O'Toole and Kolter, 1998b).

1.1.5.2 Proteomic investigations of biofilms

Although abundance of information has been acquired from genetic studies of mutants defective in biofilm formation, fewer studies have compared protein expression profiles between biofilm cells and the corresponding free- living planktonic cells. Proteomics studies have revealed major physiological differences between planktonic and biofilm mode of growth in *P. aeruginosa, P. putida*, and *Bacillus cereus* (Sauer *et al.*, 2002, Sauer and Camper, 2001, Oosthuizen *et al.*, 2002). Sauer *et al.* (2002) showed *P. aeruginosa* displayed five distinct physiologies during biofilm development based on proteomic analysis. Protein extracts separated by two-dimensional gel electrophoresis combined with reporter-gene analysis and microscopy, revealed 57 biofilm-associated proteins that were differentially expressed from the planktonic bacteria. Differentially expressed proteins were associated with metabolic processes such as amino acid, carbon, and lipid metabolism, membrane proteins and superoxide dismutase. The enhanced expression proteins were involved in oxidative damage, production of exopolymeric substances, aerobic and anaerobic metabolism, and membrane transport (Sauer *et al*., 2002).

1.1.6 Methods for investigating biofilms

a. Growth systems

Biofilms in the laboratory can be cultivated either in a model system consisting of a cultivation of defined community of one or more species, or in microcosms which is a collection of microbes from natural communities (Lemon *et al*., 2008). Four general systems have been routinely used for the study of biofilms *in vitro*: Flow cell systems, microtiter plates, floating pellicles and colonies grown on agar with complex architecture (Branda *et al*., 2005, Lemon *et al*., 2008). Biofilms have been studied

mainly in static batch culture mainly by using microtiter plates and under different flow conditions by using various flow cells.

b. Microscopy

Scanning electron microscopy (SEM) is used in the visualisation of complex structures at high magnification. Sample distortion and artefacts are common as a result of the dehydration of the sample prior to observation (Donlan & Costerton, 2002). Also, in SEMs EPS often appears as fibres rather than as thick gelatinous matrix surrounding the cells, due to the loss of water (Wimpenny *et al*., 2000). The internal cross-sectional detail of the individual microorganisms and their relationship to each other including the overall biofilm can be visualised by using transmission electron microscope (TEM). Specific polysaccharide stains like ruthenium red are used to stain the EPS in biofilms (Costerton, 1999, Wimpenny *et al*., 2000). Even though, electron microscopy has certain limitations, it is an important tool used in biofilm studies, because of its higher resolution capacity (Donlan and Costerton, 2002, Lyte *et al*., 2003).

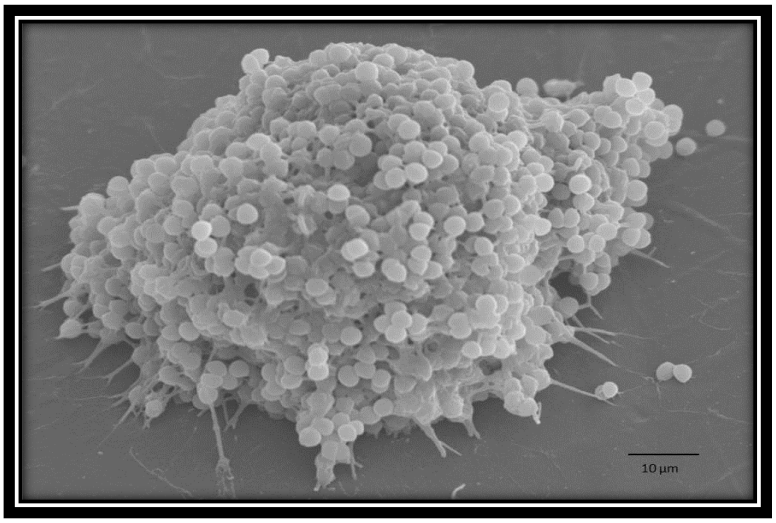


Figure 1.2 Scanning electron micrograph of an *in vitro S.epidermidis* biofilm on an intravenous catheter surface (taken from this study)

The relatively recent application of confocal scanning laser microscope (CSLM) has become a powerful tool in biofilm research. CSLM allows the *in situ* analysis of fully hydrated intact biofilms under low magnification without using chemical fixation or sample processing techniques. Both CSLM and epifluorescent microscopy needs the biofilms to be stained by using a wide range of specific fluorescent probes and nonspecific fluorescent compounds such as DAPI, Syto9, and acridine orange. The images from CSLM microscopy are assembled using image analysis softwares such as COMSTAT to generate three dimensional images with an in-depth profile of the biofilm sample (Costerton *et al*., 1995, Donlan and Costerton, 2002, Hall-Stoodley *et al*., 2004). Atomic force microscopy can also reveal the surface structures of a biofilm (Wimpenny, 2000).

c. Other biofilm characterisation methods

Molecular methods such as fluorescence *in situ* hybridization (FISH) have been used to identify individual bacterial cells. In this method, oligonucleotide probes are made to recognize 16S rRNA signatures in the biofilms and are labelled with different fluorescent dyes to visualise the cells (Donlan and Costerton, 2002).

In situ gene expression can also be studied microscopically by the use of plasmid mediated green fluorescent protein (GFP) fusion reporter constructs without fixation or staining. GFP is helpful in understanding the community structure of biofilms, and to understand the temporal and spatial distribution in expression of genes in biofilms (Hall-Stoodley and Stoodley, 2002, Wimpenny, 2000). Microelectrode experiments are used to study the physico-chemical environment of the biofilms. Dissolved Oxygen,

pH, nitrogen dioxide, special enzymes electrodes are most commonly used (Wimpenny *et al*., 2000, deBeer and Stoodley, 2004).

Nuclear resonance imaging is used to monitor flow regimes in biofilms (Lewandowski *et al*., 1993) and Fourier transform infrared red spectroscopy is used to investigate growth and attachment on a variety of solid surface materials non-destructively in real-time (Geesey *et al*., 2000).

1.1.7 Regulation of biofilm formation

Biofilm development, behaviour and population dynamics are influenced by many environmental factors and by intrinsic biological properties (Sauer and Camper, 2001, Simoes *et al*., 2007). Some common factors include extracellular components, such as flagella, pili, adhesins and fimbriae, and EPS (Donlan, 2002). Quorum sensing (discussed in section 1.2.1) nutrient metabolism and hydrodynamics are also factors associated with biofilm development (Sauer and Camper, 2001, Purevdorj *et al*. 2002, Stoodley *et al*., 2004, Simoes *et al*., 2007).

Structural components play an important role in facilitating bacterial interaction with surfaces which include flagella, pili, fimbriae, curli and outer membrane proteins (Renner and Wiebel, 2011). These structural components facilitate the cell to overcome the repulsive forces of the surface and to be attached to a surface before the permanent attachment mechanism (Sauer and Camper, 2001, Donlan, 2001). The role of EPS is discussed in section 1.6. Characteristics such as pH, nutrient levels, ionic strength, and temperature, may also play a role in the rate of microbial attachment to a surface (Donlan, 2002)

Since hydrodynamics fundamentally influences mass transfer and fluid shear stresses, biofilm behaviour may vary significantly with the flow regimen (Stoodley *et al*, 1999a, 1999b). Hydrodynamic conditions will determine the rate of transport of cells, oxygen and nutrients to the surface, as well as the magnitude of the shear forces acting on a developing biofilm. Thus, these conditions significantly influence many of the processes involved in biofilm development (Simoes *et al*., 2007). Studies have demonstrated that most laboratory biofilms grown under laminar flow conditions form towers and mushroom-shaped microcolonies, with no directionality. However, in turbulent flows, the biofilms have shown to form filamentous streamers in the downstream direction (Stoodley *et al*., 1999 a, b, Stoodley *et al*., 2000). Stoodley *et al*., (1999c) has also shown that mixed species biofilms grown in turbulent flow formed patches of ripple like structures migrating in the downstream direction (Stoodley *et al*., 1999c). When biofilms are formed in low-shear environments, they have a low tensile strength and break easily, but biofilms formed at high shear are remarkably strong and resistant to mechanical breakage (Stoodley *et al*., 2000).

Loosdrecht *et al*. (1995) suggested that the degree of biofilm heterogeneity is determined by the balance between bacterial growth rate and detachment. Also in this study slow growing organisms (e.g., nitrifiers and methanogens) were shown to form relatively flat biofilms or spherical aggregates and fast growing organisms form heterogeneous biofilms with cell clusters and streamers (Loosdrecht *et al*., 1995).

1.2 Environmental regulation of biofilms

The environmental signals which regulate initiation of biofilm formation allow bacteria to colonize its preferred niche. Environmental signal synthesized by different bacterial

species (cell to cell and interspecies communication), as well that of signals produced by eukaryotic cells (inter-kingdom communication), play an important in biofilm formation (Davies *et al*., 1998, Hall-Stoodley *et al*., 2004, Jayaraman *et al*., 2008).

Quorum sensing signal-mediated cell-cell communication between bacteria belonging to the same species, different species (interspecies), as well as interkingdom signalling is discussed below in section 1.2.1 and 1.2.2.

1.2.1 Intra- kingdom signalling - Quorum Sensing

Quorum Sensing (QS) is a phenomenon in which many species of bacteria regulate gene expression in response to increasing cell population density, by production and releasing and detection of signalling molecules. Quorum-sensing bacteria such as *P.aeruginosa*, *Vibrio* spp. produce and release acyl-homo signalling molecules (called autoinducers) that accumulate in the environment as the cell density increases. When a threshold stimulatory concentration of autoinducer is achieved, a signal transduction cascade is initiated that cause changes in gene expression which is ultimately translated into a change in behaviour of the organism (Miller & Bassler, 2001). Quorum sensing was first described by Nealson *et al*., (1970) as being involved in the regulation of bioluminescence in the symbiotic relationship of *Vibrio fischeri*, and bobtail squid (*Euprymna scolopes*). The QS molecule produced by *V. fischeri* induces the transcription of luciferase operon when the bacterial densities are above a threshold concentration. Since this initial description, homologues of QS systems have been identified involved in gene regulation in both Gram-negative and Gram-positive bacteria (Asad and Opal, 2008).

Quorum sensing can occur within a single bacterial species as well as between diverse species, serving as intra- and inter- species signalling and communication network

(Diggle *et al*., 2007). QS regulates many phenotypes in Gram- positive and Gram-negative bacteria such as biofilm formation, sporulation, biosurfactant synthesis, antibiotic production, conjugation, competence, bioluminescence, motility, clumping, DNA transfer, secretion of nutrient-sequestering compounds and virulence determinants (Bassler and Miller, 2001, Bassler, 2002, Greenberg, 2003, Diggle *et al*., 2007). The acely homoserine lactones (AHLs) and autoinducing peptides (AIPs) represent the two major classes of known bacterial cell-cell signalling molecules in Gram- negative and Gram positive bacteria respectively (Miller and Bassler, 2001). Gram-negative quorum-sensing bacteria such as *Vibrio fisheri, P. aeruginosa* communicate through AHL mediated systems, which are the products of LuxI-type autoinducer synthases. These autoinducers are detected by cytoplasmic LuxR proteins that, upon binding the partner autoinducer, bind DNA and activate transcription of target quorum-sensing genes (Bassler, 2002). Two main AHL quorum sensing systems have been identified in *P. aeruginosa*: LasR- LasI and RhIR – RhlI systems which regulate the virulence gene expression and the production of a number of secondary metabolites. The las QS system is composed of lasI, autoinducer synthase responsible for the synthesis of 3-oxo-C12-HSL (N-[3-oxododecanoyl]-L-homoserine lactone), and the signal receptor lasR. The second QS system rhl is composed of rhlI, which produces C4-HSL (N-butyrylhomoserine lactone) and the receptor rhlR, which induces gene expression by binding of C4-HSL (Davies *et al*., 1998).

Gram-positive quorum-sensing bacteria, such as *Streptococcus spp.*, *Staphylococci spp.*, *Bacillus spp.*, mainly communicate with chemically modified short autoinducer peptides (AIPs) (Bassler and Lossick, 2006, Asad and Opal, 2008). Signalling peptides are recognized by membrane-bound two-component sensor histidine kinases and signal transduction occurs by phosphorylation cascades that ultimately affect DNA binding

transcription factors responsible for regulation of target genes (Bassler and Lossick, 2006). These systems are referred to as accessory regulatory system (agr) in staphylococci and denoted by different names in other Gram positive bacteria (Asad and Opal, 2008)

Another signalling molecule known as AI-2 produced by a protein called LuxS is widely spread among both Gram- positive and Gram- negative bacteria mainly involved in interspecies communication (Bassler and Lossick, 2006). This system was first discovered in *V. harveyi* and was shown to monitor the environment of other species of bacteria (Bassler *et al*., 1997). The role of AI-2 in modulating *Streptococcus gordonii* and *Streptococcus mutans* oral cavity biofilms and disease has been well characterized (Blehert *et al*., 2003, Yoshida *et al*., 2005).

The *E. coli* autoinducer type 3 (AI-3) system shares many characteristics of the AI-2 but, in contrast to AI-2, the AI-3 system has been proposed to cross-connect with the human catecholamine stress hormones epinephrine or norepinephrine (Sperandio *et al*., 2003, Walters and Sperandio, 2006). The AI3/ Epi/NE QS system is explained in detail in section 4.1. Examples of the various signalling molecules and quorum sensing systems used by bacteria are summarised in Table 1.3.

QS signalling systems have been considered to play an important role in biofilm formation by controlling EPS production in a number of bacteria such as *Vibrio cholerae*, and *P. aeruginosa* (Davies *et al*., 1998, Hammer and Bassler, 2003, Sakuragi and Kolter, 2007). QS systems have been shown to be involved in all phases of biofilm formation and population density and the metabolic activity is controlled through quorum sensing within the mature biofilm according to the nutrient and resources availability (O'Toole *et al*., 2000, Asad and Opal, 2008). When cell densities in

biofilms become high, quorum sensing signals are used to reduce the production of EPS to permit bacteria to escape the biofilm (Davies *et al*., 1998). Quorum sensing also plays an important role in the pathogenesis of bacteria such as *P. aeruginosa* (Whitley *et al*., 1999, Davies *et al*., 1998), *S. aureus* (Tenover and Gaynes 2000). However, biofilm formation has been shown to be inhibited by both the agr system and LuxS/AI-2 signals in staphylococci. QS systems are utilised in staphylococcal biofilms in the expression of enzymes and phenol-soluble modulins that allows detachment of bacteria from mature biofilms (Li *et al*., 2008, McCann *et al*., 2008).

Since QS is an important regulator of bacterial virulence, it has been considered to be a potential drug target (Khmel and Metlitskaia, 2006, Asad and Opal, 2008). A recent study has demonstrated that *P. aeruginosa* QS system produced rhamnolipids have shown to induce necrosis of the polymorphonuclear leukocytes (PMNs) which are the major first line host defences of the host (Jensen *et al*., 2007). This is one of the studies which highlights the inhibition of QS as a target for the treatment of infections with *P. aeruginosa*.

Table 1.3: **QS model systems in bacteria** (adapted from Asad and Opal, 2008, Bassler 2002)

QS system	Signalling molecule	Receptor(s)	Example
Autoinducer 1(AI-1) LuxI/LuxR type and homologs	N-acyl-homoserine lactones	Intracellular Lux-R homologues as transcriptional co activator	*Burkholderia Vibrio Pseudomonas* spp.
Autoinducer 2 (AI-2), LuxS type	Heterocyclic furanosyl-borate	Two-component membrane receptor-cytoplasmic kinase complex	Widespread in Gram-negative and Gram-positive bacteria e.g.: Enterics, *Streptococci* spp.
Autoinducer type 3, epinephrine/norepinephrine signalling system	Norepinephrine Epinephrine AI-3	Two-component membrane-sensor kinase/ response regulator (QseBC/ EF)	Found in Gram-negative, enteric bacteria enterohemorrhagic *E.coli*, enteropathogenic *E. coli*, *Shigella*,*Salmonella* spp.
Cyclic short-peptide systems	Aromatic compound structure not yet known	Two-component sensor kinase (AgrC)-response regulator (AgrA)	Gram-positive bacteria, *Staphylococcus*, *Bacillus*, *Enterococcus*, *Streptococcus* spp.

1.2.2 Interkingdom signalling

The hormonal communication between microorganisms and their hosts has been named as microbial endocrinology and is an example of inter-kingdom signalling which involves small molecules, such as hormones that are produced by eukaryotes being sensed by bacteria (Freestone *et al*., 2008 – see section 1.3). Inter-kingdom signalling processes mediate symbiotic and pathogenic relationships between bacteria, mammalian and plant hosts (Freestone *et al.*, 2008, Pacheco and Sperandio, 2009).

It is estimated that 10^{13} human cells co-exist with 10^{14} bacteria cells belonging to about 500 species in the gastrointestinal tract. The gastrointestinal tract has an abundance of signalling molecules, prokaryotic (AI-2, AI-3, and indole) and eukaryotic (norepinephrine, dopamine) (Furness, 2006) in origin and these signals are exploited by pathogenic bacteria to activate virulence gene expression (Freestone *et al*., 2008, Pacheo and Sperandio, 2009). Many studies have shown the catecholamines stimulation of bacterial growth and virulence gene expression (discussed in section 1.3). Sperandio *et al*., (2003) demonstrated increased virulence gene expression in EHEC when grown in the presence of epinephrine and norepinephrine and proposed that the presence of adrenergic receptors in bacteria and the above hormones act as mimics of the QS signal AI-3 (discussed in section 4.1). Bacterial AHLs can stimulate responses in mammalian cells. This phenomenon was first observed using respiratory epithelial cells, in which there was a dose-dependent increase in interleukin (IL)‑8 in response to purified AHL (DiMango *et al*., 1995). Inter kingdom signalling is not only confined to catecholamines it also involves other hormones. Zaborina *et al*. (2007) observed that *P.aeruginosa* could recognize opioid dynorphins released during stress, and enhance its virulence by the increased production of QS signals. These studies provide sufficient evidence for the prevalence of interkingdom signalling, in the pathogens by recognition of host

signals by pathogenic bacteria. Since, catecholamine hormone mediated inter kingdom signalling is relevant to this study it is discussed detail below in section 1.3.

1.3 Microbial Endocrinology

Microbial Endocrinology is a recently recognised interdisciplinary research discipline that represents the intersection of microbiology, endocrinology with neurophysiology. Its objective is to examine and understand the interaction of micro-organisms with their host in health and disease states. Microbial Endocrinology has as its foundation the principle that through their long co-existence with animals and plants, micro-organisms have evolved detection systems for detecting host-associated chemicals such as hormones. These hormone-sensors enable the microbe to determine that they are within a suitable host, and that it is time to initiate the expression of genes involved in colonisation or in the case of pathogenic organisms, virulence factors. This field was founded in 1992 (Lyte and Ernst, 1992) who were the first to observe that stress-related hormones, the neurotransmitter catecholamines in particular, could directly influence bacterial growth (Lyte, 2004, Freestone *et al*., 2008, Sharaff and Freestone, 2011).

1.3.1 Stress, the immune system and infection

Hans Seyle in 1936 defined stress as "consequences of the failure of a human or animal to respond appropriately to emotional or physical threats to the organism, whether actual or imagined". Many different things can cause stress from physical such as fear of something dangerous to emotional such as worries. Stressors are agents that produce stress at anytime. Stressful experiences include physical stressors such as pathogens and

toxins, and psychological stressors such as major life events, trauma, abuse, or environmental factors such as family, neighbourhood, work place (Reiche *et al*., 2004).

The immune system and central nervous system (CNS) play a critical role in maintaining homeostasis under normal and disease conditions. When stress is perceived by the CNS, a variety of hormones, neurochemicals are released which can directly affect immune function, and harm the body (Lyte, 2004, Freestone *et al*., 2008; Reiche *et al*.,2004). Stress activates mainly two neural pathways: sympathetic-adrenal-medullary axis (SAM) and the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA and SAM axes results in systemic increases of stress hormones, such as glucocorticoids (e.g. cortisol) and the catecholamines adrenaline (epinephrine) and noradrenaline (norepinephrine), as well as a range of neuropeptides and other effectors. Sympathetic nerve fibres innervate lymphoid tissues such as the bone marrow, thymus, spleen and lymph nodes, and terminate in close proximity to lymphocytes, that possess receptors for the above mentioned effectors. Stress response system in mammals results in a bi-directional communication between the CNS, peripheral nervous system, the endocrine system and immune systems (Segerstrom and Millers, 2004, Reiche *et al*, 2004).

1.3.2 Microbial endocrinology and catecholamine stress hormones

Stress released hormones can also influence the outcome of an infection through direct effect on the infectious agent (Lyte, 2004, Freestone *et al*., 2008). Catecholamines play an essential role in a multitude of stress-related phenomena from psychological stress to trauma. Catecholamines are a group of tyrosine-derived effector compounds comprising a benzene ring with two adjacent hydroxyl groups and an opposing amine side chain. In

mammals, the catecholamine synthesis pathway is L-dopa (usually obtained from dietary sources) Dopamine → Norepinephrine → Epinephrine (Figure. 1.2) (Sharaff and Freestone, 2011, Freestone *et al*., 2008).

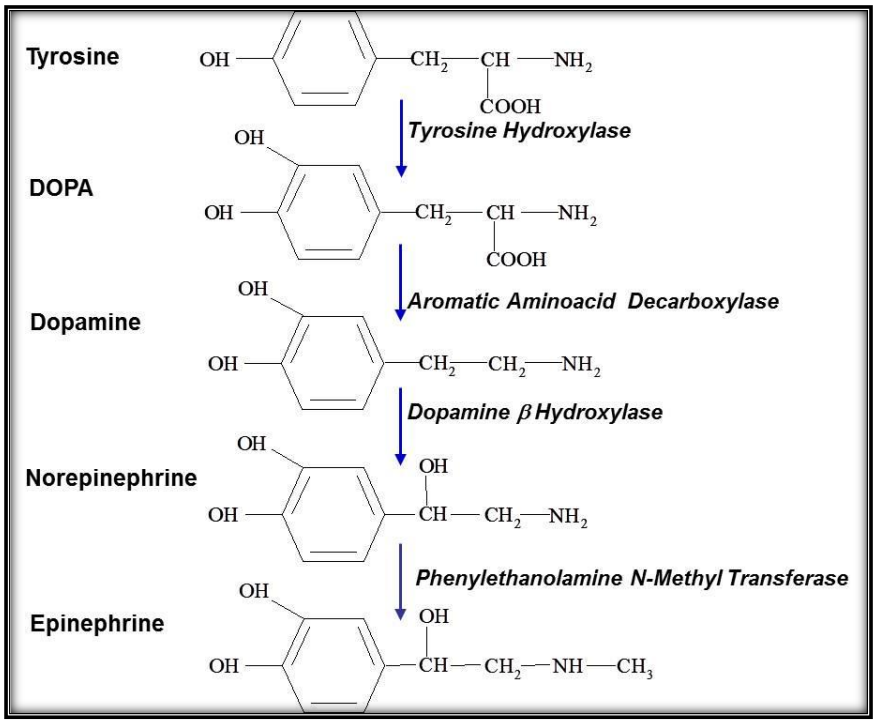


Figure 1.2 Catecholamine biosynthetic pathway: Catecholamines are effector compounds derived from tyrosine and characterized by having a benzene ring with two adjacent hydroxyl groups and an opposing amine side chain (Adapted from Sharaff & Freestone, 2011). DOPA- 3,4 dihydroxy Phenylalanine

Catecholamines mediate many different signalling phenomena in higher animals, and the catecholamine hormones adrenaline and noradrenaline facilitate the acute 'fight or flight stress response to threat (Freestone *et al*., 2008). The catecholamines also regulate behaviour, fuel metabolism, cardiovascular function and thermogenesis, collectively acting to maintain homeostasis during exposure to stressors. In response to sympathetic stimulation, epinephrine and norepinephrine are rapidly secreted into the blood stream

with marked effects on the cardiovascular system. These effects lead to increased production of blood glucose and fatty acids, providing substrates for energy production within cells. The role played by catecholamines in stress-related health problems has mainly been linked to the cardiovascular system and disorders such as hypertension, myocardial infarction and stroke (Lundberg, 2005).

Half of the norepinephrine present within mammals is synthesised and utilised within the enteric nervous system (ENS). Within the gut, norepinephrine is released from storage within sympathetic nerve fibres within the prevertebral ganglia that innervate the gut mucosa (Furness, 2006). Dopamine is produced in a subpopulation of non-sympathetic enteric neurons located within the intestinal wall (Furness, 2006, Sharaff and Freestone, 2011). Epinephrine synthesis is restricted to the central nervous system and adrenal glands. However, epinephrine is released into the bloodstream, especially during stress, acting systemically in the whole body and phenylethanolamine N-methyltransferase, required for epinephrine synthesis, is not expressed in cells of the enteric nervous system (Furness 2006, Freestone *et al*., 2008).

Both epinephrine and norepinephrine are recognized by specific adrenergic α and β receptors (AR) in mammalian cells. The adrenergic receptors are a class of heterotrimeric guanine-binding proteins (G protein) coupled receptors (GPCR) responsible for mediating the effects of the catecholamines. In 1948, Ahlquist differentiated the AR family into two major classes, alpha (α) and beta (β) adrenoceptors and currently they are further divided into α1, α2 and β (β1, β2 and β3 subclasses), each of these subtypes are coded by separate genes and display specific drug specificities and regulatory properties. The receptors are involved in the

mediation of a wide variety of physiological responses, including vasodilation and vasoconstriction, heart rate modulation, regulation of lipolysis, and blood clotting (Freddolino *et al*. 2004). Catecholamines trigger a specific response in the effector organs by interacting with the adrenoreceptors that they express. Although dopamine is a catecholamine, its receptors are in a different structural category and specific dopamine receptors are categorised as D1 and D2-like receptors (Missale *et al*., 1998). Synthetic catecholamine inotropes such as dobutamine and isoprenaline are used in intensive care settings to control heart function and regulate blood pressure (Freestone *et al*., 2008).

1.3.3 Mechanisms of catecholamine hormones effects on bacteria

Catecholamine stress hormones have been shown to be important growth stimulators for a number of microbes such as bacteria inhabiting the gastrointestinal tract, those causing respiratory infection periodontal disease and skin associated bacteria (Freestone *et al*., 1999, 2008, Neal *et al*., 2001). However, the spectrum of hormone responsive microbes is weighted towards bacteria inhabiting the gastrointestinal tract, particularly species such as *E. coli*, *Salmonella*, *Listeria*, *Campylobacter*, and *Yersinia* (Sharaff and Freestone 2011, Freestone *et al*., 2008). This may be related to the abundance of norepinephrine- and dopamine- containing nerve terminals in the enteric nervous system or ENS (Furness, 2006).

The majority of analyses of stress hormone responsiveness have been conducted *in vitro* and have used a serum or plasma-based culture supplemented with minimal salts (to reflect the host environment in which the microbe will encounter). Also used are low inoculum levels (less than 10^2 CFU/ml) which are reflective of the number of bacteria

present at the onset of an infection (Lyte, 2004, Freestone *et al*., 2008, Sharaff and Freestone, 2011). As a consequence, such medium is usually bacteriostatic through Fe limitation caused by chelation of free iron by transferrin, a serum glycoprotein with very high Fe^{3+} binding affinity (Ratledge and Dover, 2000). The iron limitation imposed by transferrin also results in poor growth of many bacterial species around 10^3-10^4 CFU/ml, depending on the microbe concerned (Freestone *et al*, 1999, 2008, Freestone and Lyte, 2009). However, if catecholamines are added, the resultant growth induction can be over 5 log orders, which means 10^2 bacteria can in 24 hrs become 10^8-10^9 CFU/ml (Lyte and Ernst, 1993, Freestone *et al*, 1999, 2008, 2009). To achieve as close to the host environment as possible, hormone concentrations used in experiments are also lower than most of those used in the literature (50 μM and higher) (Sharaff and Freestone, 2011) to reflect the levels found within the blood of inotrope medicated patients.

1.3.3.1 Catecholamines mediate access to host iron stores

The relationship between iron, bacterial growth and virulence has been known for many years, with the limited availability of free iron in the host environment presenting a major problem to the growth of most microbial pathogens. A strategy that infectious bacteria often employ to scavenge nutritionally essential iron is the production and utilisation of siderophores, that are low molecular weight catecholate or hydroxamate molecules that possess high affinity for ferric iron. However, in the presence of high affinity host ferric iron binding proteins, such as transferrin (Tf) in blood and lactoferrin (Lf) in mucosal secretions, siderophores are often ineffective at retrieving host iron (Freestone *et al*., 2008, Sharaff and Freestone, 2011). Catecholamine-facilitated iron provision from host iron binding proteins has been demonstrated for a range of Gram-

positive and Gram-negative bacteria (Freestone *et al*., 2000, 2002, 2003, 2007a, b, Lyte *et al*., 2003, Neal *et al*., 2001). Catecholamines have been shown to form complexes with transferrin and lactoferrin which weakens the high affinity Fe^{3+}-Tf complex, resulting in iron loss from the protein (Freestone *et al*., 2000, 2002). This mechanism enables bacteria that lack specific systems for acquiring transferrin and lactoferrin iron to obtain the Fe needed for growth in serum or blood (Freestone *et al*., 2008, Sharaff and Freestone, 2011). Sandrini *et al* (2010) have shown that catecholamine complex formation with transferrin and lactoferrin results in reduction of the iron, from ferric to ferrous, a valency for which transferrin and lactoferrin have a much lower affinity, resulting in rapid Fe loss from the protein. This iron theft mechanism by catecholamine explains the reason behind catecholamine growth stimulation of bacteria in serum or blood.

It has been shown in Gram negative bacteria such as *E.coli* & *Salmonella*, that both siderophores and the ferric iron transport system plays an important role in the mechanism by which catecholamine hormones stimulate growth. The catechol moiety is found both in catecholamine stress neurohormones and in many siderophores. The role of the siderophore, was to facilitate the internalization into the bacterial cell of the transferrin or lactoferrin Fe liberated by the catecholamine (Freestone *et al*., 2003). Most Fe uptake is in the ferric form but Sandrini *et al*. (2010) demonstrated that reduction of transferrin and lactoferrin Fe(III) by the catecholamines also allows incorporation of released Fe(II) by bacterial ferrous uptake systems.

1.3.3.2 Catecholamine induce growth by stimulating an autoinducer activity

Studies have demonstrated that several enteric species grown in serum based media containing catecholamines induce the production of a novel growth inducer. . This inducer has been termed as the Norepinephrine induced- auto inducer (NE-AI), a heat stable and highly cross species acting. The NE-AI induces bacterial growth to a similar magnitude as the catecholamines (Lyte *et al*., 1996, Freestone *et al*., 1999, Lyte and Freestone, 2008).

1.3.3.3 Catecholamine effect on bacterial virulence

Many studies have demonstrated the effect of stress-related neuroendocrine hormones on bacterial virulence *in vivo* and *in vitro*. Some of the studies are briefly discussed below: Norepinephrine has been shown to increase the production of shiga toxins by *E. coli* O157:H7 (Lyte *et al*., 1996) and the intestinal mucosa adherence and enteropathogenicity in a bovine ligated ileal loop model of infection (Vlisidou *et al*., 2004). The production of the K99 pilus adhesin by the bovine enterotoxigenic *E. coli* strain B44 which aids in attachment of the bacterium to the epithelial cell surface, was also shown to be increased over 1600-fold (Lyte *et al*., 1997). NE also induced enterotoxin-production in *Salmonella* Typhimurium in a similar manner as shiga toxin (Chen *et al*., 2003). Bansal *et al*. (2007) demonstrated that in addition to host cell attachment, *E. coli* O157:H7 showed a positive chemotactic response to norepinephrine and epinephrine. Toscano *et al*. (2007) showed the pre-treatment of *Salmonella* Typhimurium with NE altered its tissue dissemination in a porcine model of infection. The gene expression profiles obtained from catecholamine-treated bacteria support the view that exposure to stress hormones enhances expression of genes involved in

bacterial pathogenicity (such as motility, iron acquisition, and epithelial cell attachment) (Bansal *et al*., 2007, Dowd, 2007). Recently, Peterson *et al*. (2011) showed in vitro that stress hormone exposure enhanced the horizontal gene transfer efficiencies of a conjugative plasmid from a clinical host strain of *Salmonella* Typhimurium to an *E. coli* recipient.

1.3.4 Catecholamines and biofilm formation

Medical devices such as intravascular catheters, has been identified as the commonest source of infection among patients in intensive care (Rello *et al*., 1994, Rosenthal *et al*., 2006). However, ICU patients are so susceptible to infections, despite intensive antibiotic prophylaxis. Lyte *et al*. (2003) suggested a new theory for the ICU device related infections using microbial endocrinology as a tool (Lyte *et al*., 2003). Approximately 50% of patients in intensive care units (ICU) receive catecholamine inotropic support during their hospital stay (Smythe *et al*., 1993) For example dobutamine is used in the treatment of congestive heart failure, epinephrine for the treatment of anaphylactic shock, dopamine to support renal function (Freestone *et al*., 2010). The majority of catheter-associated nosocomial infections are caused by coagulase negative *staphylococci*, though *P. aeruginosa* is also problematic. The coagulase-negative *staphylococci* in particular *S. epidermidis* pose a serious threat for intensive care unit patients because of their ability to colonise and form biofilms within intravenous catheters (Lyte *et al*., 2003). Lyte *et al*. (2003) have shown that catecholamine stress hormones (such as epinephrine and NE) and structurally similar drugs (such as dobutamine) not only accelerate planktonic growth of *S.epidermidis*, but also enhance biofilm formation on catheter-grade plastics (Lyte *et al*., 2003). This discovery has led to the suggestion that the drugs which are used in the hospital setting

may serve as environmental factors leading to bacterial biofilm formation of indwelling medical devices, and subsequent hospital acquired infection.

1.4 Organisms Investigated

1.4.1 *Pseudomonas aeruginosa*

P. aeruginosa (family *Pseudomonadaceae*), an aerobic, motile, Gram-negative rod, adapts to and thrives in many ecological niches, from water and soil to plant and animal tissues. *P. aeruginosa* has very minimal nutritional requirements and known for its metabolic versatility. It does not require growth factors and able to use a wide variety of organic compounds as carbon sources. *P. aeruginosa* produces a variety of pigments including a fluorescent yellow-green pyoverdin as well as a blue-green pigment pyocyanin (Todar, 2009).

P. aeruginosa is an opportunistic pathogen, causes serious infections in immunocompromised patients including severely burned patients, HIV-infected individuals, cancer patients undergoing chemotherapy, and patients suffering from cystic fibrosis (Lyczak *et al*., 2000, Pollack, 2000). It causes community-acquired infections such as ulcerative keratitis, otitis externa, skin and soft tissue infections (including diabetic foot infections) and noscomial infections which include pneumonia, urinary tract infections, bloodstream infections, surgical site infections (Driscoll *et al*., 2007). *P. aeruginosa* is responsible for about11-13.8% of all nosocomial infections in different ethnic communities worldwide (Bandara *et al*., 2010). The pathogenesis of pseudomonal infections is complex and multifactorial involving many virulence factors. Pollock (2000) has proposed three stages in pathogenesis 1) bacterial attachment and colonization, (2) local infection, and (3) bloodstream dissemination and systemic disease. *P. aeruginosa* has both cell-associated and extracellular virulence factors (Table 1.4) which lead to extensive tissue damage, bloodstream invasion, and dissemination. Many of these factors are controlled by quorum sensing systems.

Two main based quorum sensing systems have been identified in *P.aeruginosa*: LasR-LasI and RhIR – RhlI systems which regulate the virulence gene expression and the production of a number of secondary metabolites. The las QS system regulates lasB elastase expression and is required in the production of extracellular virulence factors such as LasA protease and exotoxin A. The second QS system *rhl* regulates the expression of the rhlAB operon responsible for rhamnolipid production. The rhl QS system is also necessary for production of virulence factors such as LasB elastase, Las A protease, pyocyanin and alkaline protease and in the regulation of the expression of rpoS, which encodes a stationary phase sigma factor involved in the regulation of various stress-response genes (Van Delden *et al*., 1998, Schuster and Greenberg, 2006). *P. aeruginosa* has an additional QS regulatory pathway termed the *Pseudomonas* quinolone signal (PQS) system which regulates rhl dependant genes during stressful conditions (Diggle *et al*., 2003).

P. aeruginosa is resistant to many antibiotics such as β lactams, macrolides, tetracyclines, co-trimoxazole (trimethoprim/sulfamethoxazole) and most fluoroquinolones and sensitive to aminoglycosides (tobramycin, gentamicin), third fourth generation cephalosporins, and carbapenems. *P. aeruginosa* nosocomial infections have shown an increasing trend of antimicrobial resistance and multidrug resistance. General mechanisms attributing to the antibiotic resistance include blockade of entry, active efflux from the cell by multidrug efflux pumps, β lactamase activity, target structure alteration, down regulation of outer membrane porins (Schweizer, 2003, Driscoll *et al*., 2007).

Table 1.4 Common virulence factors of *P.aeruginosa* (Sadikot *et al*., 2005, Driscoll *et al*., 2007, Wilson and Dowling, 1998)

Virulence factor	Function
Cell associated factors	
Flagellum	Motility
Pili	Adherence to epithelium and twitching motility (type 1V)
Alginate/Biofilms	Adherence, protection, survival strategy
Lipopolysaccharides (LPS)	mediates host pathogen interaction
Extracellular factors	
Exoenzyme A	ADP ribosylation of elongation factor 2 and inhibits host cell protein synthesis, tissue damage, bacterial invasion, and immunosuppression
Type III secretion system Exoenzyme S ,U, T, Y	Allows injection of toxins into the host cell direct tissue destruction (in lung infection), cytotoxicity and important for bacterial dissemination
Haemolysins Phospholipase C Rhamnolipid	 Breaking down lipase and lecithin, tissue damage Acts as biosurfactant. Breaks down lipases and lecithin with Phospholipase C. Aids in mucociliary transport and ciliary function of human respiratory epithelium.
Pyocyanin(blue pigment)	Inhibition of cell respiration, ciliary function, epidermal cell growth, and prostacyclin release, disruption of calcium homeostasis, inactivation of catalase
Pyoverdin, Pyochelin	Siderophores Iron acquisition

1.4.2. Enterohaemorrhagic *E. coli* (EHEC) O157:H7

Enterohaemorrhagic *E.coli* was first recognized as a cause of human disease in 1982, during an outbreak investigation of hemorrhagic colitis associated with consumption of undercooked hamburgers (Riley *et al*., 1983). EHEC strain O157:H7 is a major food-borne pathogen causing bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhoea and haemolytic uremic syndrome (HUS) in humans worldwide. The principal reservoir of EHEC is the intestine of healthy cattle and a wide variety of food items have been associated with disease, including undercooked ground beef or pork, contaminated water, sandwiches, unpasteurized milk, apple juice and vegetables. EHEC strains of the O157:H7 serotype is the most important EHEC pathogen in North America, the United Kingdom and Japan, but several other serotypes are prominent in other countries (Kaper *et al*., 2004).

EHEC is a Gram negative, motile, rod shaped, facultative anaerobic bacterium, characterized by the production of verotoxin or Shiga toxins (Stx). Serotype O157:H7 colonizes the human colon and is most often responsible for severe food borne illnesses worldwide. There are many serotypes of Stx-producing *E. coli*, but only those that have been clinically associated with haemorrhagic colitis (HC) are designated as EHEC. It is highly virulent with a low infectious dose of 10 - 100 cells: EHEC adhere to host epithelia through fimbriae and are moderate invaders of mucosal cells (Kaper *et al*., 2004).

EHEC infections progress through a three-step mechanism, the first step involves adhesion of bacteria to host cells and then the formation of microcolonies, protein translocation by type III secretion and pedestal formation (Kaper *et al*., 2004, Bansal *et al*., 2007).

EHEC forms attaching and effacing (AE) on intestinal epithelial cells. The AE lesion is characterized by the destruction of the microvilli and the rearrangement of the cytoskeleton to form pedestal-like structure that cups bacterium individually. Three major virulence factors of *E. coli* O157:H7 have been identified, which include a pathogenicity island called the Locus of Enterocyte Effacement (LEE), Shiga toxins (Stx) and the plasmid (pO157) encoded enterohaemolysin gene (E-hlyA) that codes for a pore-forming cytolysin (Yekta *et al*., 2010).

LEE (locus for enterocyte effacement) encodes virulence factors necessary for AE lesions LEE contains 41 genes arranged in five major operons: LEE1, LEE2, LEE3, tir (LEE5) and LEE4. Operons LEE1, 2 and 3 encode the majority of the components of the type III secretion apparatus tir/LEE5 encodes the bacterial adhesin intimin and the translocated intimin receptor (Tir) and LEE4 encodes the proteins EspA, EspB and EspD, which are secreted by the type III secretion system. Injection of bacterial virulence factors via the type III secretion system and binding of intimin to Tir leads to a strong interaction between bacteria and host cells (Kaper *et al*., 2004).

Shiga-like toxins are functionally identical to toxins produced by virulent *Shigella* species. Shiga toxin production is encoded by Shiga toxin genes (stx1 and stx2). Shiga-like toxins are iron-regulated toxins which inactivate 60S ribosomal subunits and cease the host protein synthesis. Stx is produced in the colon and travels by the bloodstream to the kidney, where it damages renal endothelial cells and blocks the microvasculature which leads to HUS, which is characterized by haemolytic anaemia, thrombocytopenia and fatal acute renal failure. Stx also mediates local damage in the colon, which results in bloody diarrhoea, haemorrhagic colitis (Kaper *et al*., 2004).

Treatment of the disease includes fluid replacement and blood pressure support to prevent death from dehydration, however, patients recover without treatment in five to 10 days. Treatment with antibiotics may induce shiga toxin production and lead to a higher risk of HUS. Haemolytic-uremic syndrome is usually treated in an intensive care unit by blood transfusions and kidney dialysis (Walterspiel *et al*., 1992).

1.4.3 *Salmonella* Typhimurium

Salmonella Typhimurium is a Gram-negative facultative rod belonging to family Enterobacteriaceae and genus *Salmonella*, subspecies enterica. *Salmonella* Typhimurium is an important intracellular pathogen that infects a broad range of hosts. In humans, Typhimurium causes gastroenteritis characterized by vomiting, diarrhoea, and abdominal cramps. Typhimurium food borne illnesses occurs due to the consumption of contaminated food such as poultry, pork, eggs, and dairy products. Chickens are the primary reservoir and asymptomatic carriers of the infection (Dieye *et al*., 2009).

S.Typhimurium has at least 80 different virulence genes and a large part of these genes are clustered in *Salmonella* pathogenicity islands (SPIs) and small clusters are found in pathogenicity islets. Five SPIs have been identified. *Salmonella* Pathogenicity islands (SPI) 1 and 2 are the major virulence determinants which encode type III secretion systems (T3SS) that form syringe-like structures on the membrane of the bacteria that enable the injection of effector proteins right into the host cytoplasm. (SPI) 1 involves in the invasion of epithelial cells and intestinal disease, SPI-2 SPI-3, and SPI-4 are required for intracellular survival in macrophages, systemic persistence and disease (Santos *et al*., 2003, Coburn *et al*., 2005).

Salmonella primarily colonize the small intestine and penetrate the intestinal barrier. Invasion of the intestinal mucosa results in an extrusion of the infected epithelial cells into the intestinal lumen and a destruction of microvilli, which leads to a loss of absorptive surface. The invasions of epithelial cells stimulate the production of pro inflammatory cytokines which stimulate the influx of polymorphonuclear leukocytes into the infected mucosa and massive neutrophil transmigration into the intestinal lumen (Ohl and Miller, 2001, Zhao, 2002).

Treatment is not necessary for the *S.*Typhimurium gastroenteritis; however, if the infection persists, it can be treated with antibiotics such as ampicillin, gentamicin, trimethoprim/sulfamethoxazole, ceftriaxone or amoxicillin. Antimicrobial resistance and multidrug resistance are observed in certain strains (Ohl and Miller, 2001).

1.4.4. *Staphylococcus epidermidis*

Staphylococcus epidermidis is a Gram-positive, facultative anaerobic, coagulase-negative (CoNS) staphylococcus that normally colonises the human skin and mucosa. It is also an opportunistic pathogen which causes serious nosocomial infections especially among immunocompromised and critically ill patients (Nilsson, *et al*. 1998, McCann *et al*., 2008). It is the main causative agent of biomaterial associated infections on central or peripheral intravenous catheters, joint prostheses, vascular grafts, surgical site, , cerebrospinal fluid shunts, and cardiac devices (Rupp *et al*., 1995, O'Gara and Humphreys, 2001, Bayston *et al*., 2000, McCann *et al*., 2008). *S. epidermidis* has been ranked as the premier major cause of nosocomial infections according to Centres for disease Control (CDC) National Nosocomial Infections Surveillance (2004).

S. epidermidis strains are highly diverse in clinical and geographical origin and 74 sequence types have been identified by multilocus sequence typing (Miragaia *et al*., 2007). They also consist of sodium ion/proton exchangers to withstand high salt concentration and transport systems for osmo-protection (Otto, 2009).

The ability to form biofilms on devices is the main virulence factor of *S.epidermidis*. Bayston and Penny (1972) are the first to report that many *S. epidermidis* strains of Baird-Parker's SII biotype attached to surfaces *in vitro* and observed similar growth on CSF shunts (Bayston and Penny, 1972, Christensen *et al*., 1982). Two stages of staphylococcal biofilm formation have been described (Mc Cann *et al*., 2008). The first stage involves attachment of cells to a surface; with the indwelling medical device or with host-derived products such as fibronectin or fibrinogen that initially coat the device. The adhesion to uncoated surface is dependent on cell surface hydrophobicity, which is mediated by certain proteins such as AtlE (a bifunctional adhesin and

autolysin) and the Bap (Bhp) protein (Otto, 2009). The adhesion to host protein matrix covered surfaces are mediated by surface proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) such as SdrF, SdrG and SdrH (Mc Cann *et al*., 2008, Otto, 2009). In the second stage the cells multiply and form mature biofilm consisting of many layers. This stage is associated with the production of extracellular factors, including the polysaccharide intercellular adhesin (PIA/PNAG) extracellular matrix and specific proteins (Bap and Aap) and accessory macromolecules (such as teichoic acids) aid intercellular aggregation. The icaADBC (intercellular adhesion) gene locus of *S.epidermidis* contains genes essential for PIA synthesis (Mack *et al*., 1999, Otto, 2009). Detachment of cells from the established biofilm may then allow staphylococci to spread and colonize new sites (Yarwood and Schlievert, 2003).

Other virulence factors of *S. epidermidis* include enzymes such as metalloproteases, cysteine proteases, serine proteases, lipases and antimicrobial proteins (Vyong *et al*., 2002). Toxin production is mostly limited to phenol soluble modulins (PSMs) in contrast to *S.aureus* which produces a variety of toxins. PSMs are short α-helical peptides which have pro-inflammatory and cytolytic functions. There are two main PSMs identified in *S.epidermidis*, cytolytic δ-toxin and non-cytolytic β-type PSMs. The δ-toxin has been suggested to be involved in necrotizing enterocolitis in neonates (Otto, 2009).

Two QS systems have been identified and characterised in staphylococci the luxS QS system and the accessory gene regulator (agr) system. The agr system is encoded by the agr operon (accessory gene regulator) and comprises a two-component signal transduction system (AgrA and AgrC). Studies have shown that the activation of the

two QS systems repress biofilm growth while disabling the regulatory system encourages biofilm formation (Vyong and Otto, 2003, McCann *et al*., 2008): The *luxS* system represses biofilm development by down regulating PIA production; the *agr* system down regulates AtlE production. The *agr* locus controls the expression of toxins and secreted virulence factors and the interaction with the immune system (Kong *et al*., 2006).

S.epidermidis has developed resistance to many common antibiotics such as methicillin rifampicin, fluoroquinolones, gentamicin, tetracycline, chloramphenicol, erythromycin, clindamycin and sulphonamide . Resistance to methicillin is encoded on mecA gene in the staphylococcal cassette chromosome mec (Otto, 2009).

1.5 Aims and Objectives

Patients within ICUs are recognised as being highly susceptible to infections, particularly by biofilm-associated bacteria. Why this is so is not fully understood, but what is known is that acutely ill patients are are routinely medicated with catecholamine inotropes to maintain heart and kidney function (Smythe *et al.,* 1993). Previously, it has been shown that catecholamines enhance biofilm formation by *S. epidermidis* (Lyte *et al*., 2003). The overall aim of this study is therefore to investigate if catecholamine inotropes are acting as an environmental signal to influence the ability of infectious bacteria to form biofilms.

Objectives

Three classes of bacteria enteric pathogens (*E .coli*, *S.* Typhimurium), respiratory (*P. aeruginosa*) and skin associated pathogen (*S. epidermidis*) are investigated to understand the influence of catecholamines on their growth, virulence, biofilm formation. The effect of inotropes on biofilm production will be studied at both the microscopic and molecular level in different host environments and medical device materials.

Chapter Two

General Materials & Methods

2. Materials and Methods

2.1 Materials

2.1.1 Bacterial Strains

Escherichia coli

Entero Haemorrhegic *E. coli* (EHEC) O157:H7 85-170 Nal^R is a spontaneous nalidixic acid resistant stx1- and stx2-lacking derivative of strains 84-289 (Vlisiduo *et al*., 2004) and the Δqse mutants of this strain were gifted by Professor Mark Stevens of the Institute of Animal Health, Compton, UK. The strains contained the following gene fusions: JH3008 (a promoterless gfp+), JH3009, JH3010 and JH3016. EHEC adrenergic sensor genes, qseC and qseE, were mutated by λ Red recombinase-mediated integration of linear PCR products. *qseCE* mutant was prepared from ΔqseC by a second round of λ Red mutagenesis and the Kanamycin cassette excised to produce ΔqseCE. For analysis, the EHEC strains and their wildtype parent were cultured in Luria-Bertani (LB) medium or serum-SAPI medium supplemented with antibiotics where appropriate at the following concentrations: ampicillin, 100 μg ml^{-1}, tetracycline 25 μg ml^{-1} and kanamycin 50 μg ml^{-1}.

***Salmonella* Typhimurium**

The *Salmonella enterica* serovar Typhimurium bovine strain ST4/74 and the Δqse mutants were obtained from Professor Mark Stevens of the Institute of Animal Health, Compton. The bovine *S. Typhimurium* isolate 4/74 and strains were cultured in Luria-Bertani (LB) medium or serum-SAPI medium supplemented where appropriate with antibiotics at the following concentrations: nalidixic acid (Nal, 20 μg ml^{-1}), ampicillin (Amp, 100 μg ml^{-1}) and kanamycin (Kan, 50 μg ml^{-1}). The *S. Typhimurium* isolate 4/74

strains contained the following gene fusions: JH3008 (a promoterless *gfp+*), JH3009, JH3010 and JH3016. *S.* Typhimurium orthologues of the EHEC adrenergic sensor genes, *qseC* and *qseE,* were mutated by λ Red recombinase-mediated integration of linear PCR products (Pullinger et al, 2010). The *qseCE* mutant was prepared from the EHEC Δ*qseC* by a second round of λ Red mutagenesis and the kanamycin cassette excised to produce ΔqseCE (Pullinger *et a.l,* 2010).

Pseudomonas aeruginosa

The *P. aeruginosa* strains used in this study are clinical isolate (CI) from a patient with pneumonia and *P. aeruginosa* reference strain (UCBPP-PA14) were obtained from reference culture collection, from the Leicester Public Health Laboratory, Leicester Royal Infirmary, UHL Hospitals Trust, Leicester, UK.

Staphylococcus epidermidis

The biofilm-positive *S epidermidis* strain RP62A (ATCC 35984) was purchased from LGC Standards, Middlesex, UK.

2.1.2 Culture preservation

Stock cultures of each species of bacteria were maintained in tryptic soy broth or Luria broth, by adding 25 % (v/v) sterile glycerol, dividing into 1 ml aliquots and freezing at -80°C. An aliquot of this stock was thawed and to ensure validity of the strains reactivated, culture on plates once every 24 hours for three days before use in the experiments.

2.1.3 Neurochemical preparation

Catecholamines were purchased from SIGMA, Poole, UK; L(-)-Norepinephrine-(+)-bitartrate salt monohydrate (NE) A-9512, dopamine hydrochloride H8502 (Dop), Epinephrine hydrochloride E4642 (Epi). All catecholamines were prepared shortly before use as a 5-mM stock solution in nano pure water, and then sterile filtered through a 0.2-µm pore size syringe unit filter.

2.1.4 Non-catecholamine inotropes

Vasopressin (20 Units/ml, 600 Units/mg) and phenylephrine (10 mg/ml) were obtained from the Department of Pharmacy, Leicester Royal Infirmary, University Hospitals of Leicester, UK.

2.1.5 Proteins

Human holo-transferrin (Siderophilin, iron-saturated) (T-4132), human transferrin (Siderophilin, partially saturated) (T-3309) and human apo-transferrin (T-4382) were purchased from SIGMA, Poole, UK. Peroxidase conjugated ChromPure human transferrin (Cat.no 009-030-050) was purchased from Jackson ImmunoResearch laboratories,Suffolk, UK.

2.1.6 Stains & Dyes

Crystal Violet biofilm stain was obrained from (BDH Chemicals, UK)

Congo Red (BDH Chemicals, UK)

Commassie brilliant blue (Sigma Aldrich, Poole, UK)

FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Catalog no. L10316) Invitrogen Molecular Probes, Inc, Fisher Scientific, Loughborough, UK.

SYPRO Ruby Biofilm Matrix stain (Catalog no. F10318), Invitrogen™ Molecular Probes, Inc.

Prestained SDS-PAGE Standards, broad range (161-0318), BIO-RAD

All routine laboratory reagents and chemicals were purchased from Sigma Aldrich (Poole,UK) Biorad (UK), Fisher Scientific (UK) and GE health care (UK) 'unless' otherwise stated.

2.1.7 Culture Media

Culture media and media components were purchased from Oxoid, UK

Table 2.1 Culture media used in the study

Culture Media	**Ingredients**
Luria Bertani broth Luria Bertani Agar-	1% ($^{w}/_{v}$) tryptone, 0.5 % ($^{w}/_{v}$) yeast extract, and 0.5 % ($^{w}/_{v}$) NaCl, adjusted to pH 7.0 with 1 M NaOH Luria broth solidified with 1.5% (w/v) agar
serum-SAPI (Standard American Petroleum Institute) minimal medium	6.25 mM NH_4NO_3, 1.84 mM KH_2PO_4, 3.35 mM KCl, 1.01 mM MgSO4 and 2.77 mM glucose, pH 7.5, supplemented with 30% (v/v) adult bovine serum) (Lyte, 1993b).
M9 Media	5x M9 salts ($Na_2HPO_4.7H_2O$, KH_2PO4, NaCl, NH_4Cl), 1M $MgSO_4$, 1M $CaCl_2$, 0.4% glucose
Dulbecco Modified Eagles Medium	DMEM (D596, Sigma, Poole, UK)
SAPI-Tris medium	SAPI supplemented with 100mM Tris-HCl pH 7.5 (Freestone et al., 2001)
Tryptone Soya Broth & Tryptone Soy Agar (TSB and TSA)	TSB, TSA(CM0129, CM0131-)
T- Broth	1% Tryptone 40 µg/mL Congo-red
Plasma SAPI	SAPI medium supplemented with platelet rich plasma.

SAPI and Luria Broth culture media were made using nano pure water and sterilised by autoclaving at 121 ^{o}C for 15 minutes; they were stored at room temperature until required. Bacterial cultures for experiments, unless stated other wise, were grown in LB overnight at 37°C with shaking (180 rpm) in an orbital incubator. Serum-SAPI-growth analyses were cultured statically at 37°C in a 5 % CO_2 humidified incubator.

2.1.8 Buffers and Solutions

Table 2.2 Buffers and solution used for proteomics analysis

SDS PAGE	
Resolving gel (12%)	npH_2O 4.9 ml Acrylamide 6.0 mL 1.5 M Tris HCl pH 8.8) 3.8 ml, 10% SDS 0.15 ml, 10% Ammonium per sulphate-0.15 ml, TEMED- 0.006 ml (Sigma,)
Stacking gel (5%)	npH2O 2.9 ml, Acrylamide 0.67 mL, 1.0 M Tris HCl pH 6.8) 0.5 ml, 10% SDS 0.04 ml, 10% Ammonium per sulphate-0.04 ml, TEMED- 0.004 ml
Sample loading buffer (Lammeli) (4X)	Tris HCl pH (6.8) 0.25 M, Glycerol 30 %, SDS 8%, DTT (dithiothreitol) 0.3M, Bromophenol blue 0.02%
Phosphate buffered Saline	Na_2HPO_4, Na_2PO_4, NaCl
Envelope buffer	10 mM Tris HCl (pH 7.5)
Tris EDTA buffer	10 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.3mg/ ml Phenyl Methyl Sulfonyl Fluoride PMSF (Sigma, Poole, UK)
SDS PAGE electrophoresis buffer	Tris base, Glycine, 10% Sodium dodecyl sulphate
Coommassie Blue PAGE Staining Solution	Methanol 40%, Glacial acetic acid 10%, 0.1% Commassie brilliant blue
PAGE Destaining solution	Methanol 40%, Glacial acetic acid 10%,

All the SDS PAGE buffers and solutions were prepared in nanopure water according to the methods of Sambrook et al. (1989).

2.2 Methods

2.2.1 Catecholamine growth assay experiments

Overnight cultures grown in Luria Broth (LB) were serially diluted to around at 50-100 CFU/ml into serum-SAPI medium (these low initial numbers of bacteria are intended to mimic the likely infectious doses occurring *in vivo*) (Freestone *et al.*, 1999, 2009). Serum-SAPI was supplemented with the following catecholamines at 5, 50 and 100 μM final concentrations: dopamine, norepinephrine and epinephrine. Controls consisted of the solvent used to prepare the catecholamines (water). Precise values of initial inocula were determined using plate counting. Cultures were incubated statically at 37°C in a 5% CO_2 humidified incubator for 18-24 hrs, and the final growth levels were determined by mixing of cultures, followed by serial dilution in PBS and plating onto Luria agar. All catecholamine responsiveness assays and plate count measurements were carried out in duplicate, and all sets of experiments performed on at least three separate occasions. The term `growth' refers to CFU/ml. (Freestone *et al.*, 1999).

2.2.2 Bacterial motility assays

Bacterial swimming motility assays were performed using DMEM or LB solidified with 0.3 % agar using adaptations of previous methodologies by O'Toole and Kolter (1998) and Merighi *et al.*, (2009). Bacteria were inoculated from overnight cultures which were grown at 37°C with shaking in LB, and then grown to exponential phase by diluting the inoculum to OD_{600} 0.1, in fresh LB and growing on for a further 2 hrs. This

culture was then stab inoculated into the 0.3% agar DMEM or LB agar plates containing 5 μM and 50 μM additions of norepinephrine, epinephrine, dopamine or an equivalent amount of water (control). Plates were incubated statically at 37°C for 16 hrs, and motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation and the diameters of zones of growth recorded (O'Toole and Kolter, 1998, Merighi *et al*., 2009).

2.2.3 Analysis of bacterial biofilm formation

2.2.3.1 Fluorescence microscopy analysis

Bacteria were grown overnight in LB, and where appropriate diluted into 1:100 in LB, TSB, DMEM, serum-SAPI or plasma SAPI media with and without the addition of catecholamine concentrations as indicated in individual experiments. The cultures were cultured in 150 μl volumes in a 96 well Polystyrene microtitre flat bottom plate (Corning Costar, Cat. No. 450653), and incubated statically at 37°C for up to 48 hrs. After incubation cultures were removed and the wells were stained with FilmTracer™ LIVE/DEAD Biofilm Viability kit or with SYPRO ruby biofilm matrix stain according to the manufacturer's protocol. Stained wells were observed under NIKON Ti inverted fluorescence microscope at 40x objective at 480 exitation and 500 nm emission spectra (live dead stain) and 450 excitation and 610 emission spectra (matrix stain) using FITC or TRITC filters. The biofilms were visualised and recorded using a FASTEC IMAGING camera. The resulting TIFF image files were analysed using Image J software.

2.2.3.2 Fluorescence microscopy analysis and quantification of biofilm coverage

A NIKON Eclipse Ti 2000 inverted microscope connected to NIS elements (Nikon's Original imaging software) was used to image capture bacterial biofilm formation in 96 well plates under different analysis modes (fluorescence and phase contrast). NIS-Elements (Nikon's Original imaging software) provide an integrated control of the microscope, cameras, components and peripherals of the microscope and allow the programming of automated imaging sequences. These analyses were on image analysis programmes used by Dr. Claire Smith, Leicester Royal Infirmary for scanning the culture wells of 24 or 96 well plates.

The planktonic phase of the bacterial cultures was removed and the biofilm bacteria within the plate wells were stained with FilmTracer™ LIVE/DEAD Biofilm Viability kit or with SYPRO ruby matrix stain. The stained wells were observed under a 10x objective and scanned using a NIKON Eclipse TE 2000 inverted microscope Figure 2.1) using appropriate filters for fluorescence (FITC/TRITC). Scanned images of the microscopic fields of the wells were merged using the Nikon NIS elements software.

Merged images were further analysed using the ImageJ software to quantify the area coverage of fluorescent emission of the biofilm bacteria. The area, integrated density, mean gray value of each well biofilm coverage was measured using Image J functions. "Integrated Density" is defined as the sum of gray value pixel within an image. Total image fluorescent intensities per bottom surface of the wells was measured as the sum of the integrated densities of a selection, minus the background (Ma and Bryers, 2010, Burgess *et al*., 2010).

Integrated density readings (quantification of light emitted) of the green fluorescent (biofilm stain) or red fluorescent (SYPRO Ruby) images was measured and plotted to quantify biofilm formation of each test organism to study the differences in biofilm formation with and without the catecholamines.

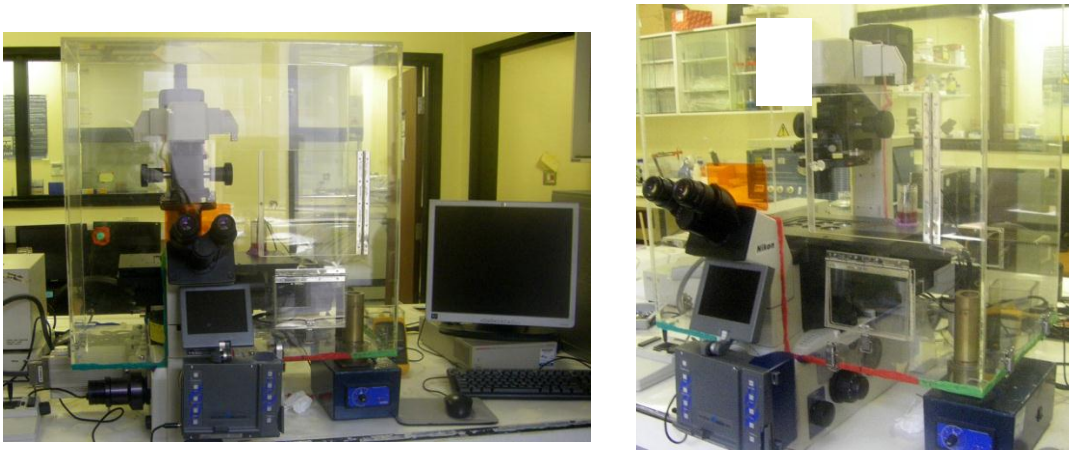


Figure 2.1 NIKON Eclipse TE 2000 inverted fluorescent microscope setup
This microscope was used for microtitre plate biofilm formation capture and analysis.

2.2.3.3 Quantification of bacterial biofilm formation

Microtitre plate biofilm formation assay

Bacterial biofilm formation was investigated by measuring attachment to polystyrene plastic 96 well microtitre plates (Nunc, tissue culture treated) according to the method of O'Toole et al. (1999) and Stepanovic et al. (2007). The test bacteria were grown overnight in LB or TSB, and then diluted to an OD_{600}~0.1 in DMEM or TSB or serum-SAPI or Plasma SAPI medium supplemented with and without different concentrations of catecholamine inotropes (dopamine, norepinephrine, epinephrine). The optimum incubation period for each bacterial species was determined empirically; all biofilm formation analysis cultures were incubated statically at 37°C for 24, 48 hrs in a 5% CO_2 humidified incubator. After incubation, planktonic bacteria were removed and

planktonic cell levels measured by recording the optical density at 595 nm before the removal of the medium. The wells were washed gently with PBS, and then 0.1 % crystal violet stain was added and the plate incubated at room temperature for 15 minutes. The stain was carefully removed, the cultures washed 2-3 times with water to remove the excess stain, and adherent crystal violet stain eluted with 20:80 v/v acetone: ethanol for *E.coli* and *S.Typhimurium*, with 95% ethanol for *P.aeruginosa* or with 33% glacial acetic acid for *S.epidermidis*. Biofilm formation was was measured at 595 nm using a microplate reader (BIORAD). Correction for background staining was made by subtracting the value for crystal violet bound to un-inoculated media controls (O'Toole *et al*., 1999, Stepanovic *et al*., 2007).

2.2.3.4 Scanning electron microscopy of bacterial biofilm formation to clinical plastics

Bacterial biofilm formation on clinical plastic polymers was performed using sections of sterile polyvinylchloride (PVC) endotracheal tube (ET-oral/nasal 9,6 ref 109-70) and polyurethane intravenous catheters (Introcan Safety® IV Catheter, 16G x 2", B. Braun, Germany). To ensure similarity in growth rates between control and catecholamine-treated cultures, *P. aeruginosa or S.epidermidis* was added to culture media at a higher inoculum of 10^5 CFU per ml to serum-SAPI medium with the catecholamine additions shown in the text. The bacteria were then incubated at 37°C in a 5% CO_2 humidified incubator for 48 hrs. The endotracheal tube sections were aseptically removed using forceps, washed three times with 2 ml of sterile PBS, and then once with 2ml of 0.1M sodium phosphate buffer pH 7·2, to remove residual serum protein (which can cause the appearance of flakes on scanning electron micrographs). Biofilms on the endotracheal tube and IV sections were fixed with cold 2.5% buffered glutaraldehyde followed by

dehydration in a graded series of ethanol to 100%, followed by infiltration with hexamethyldisilazane before air drying. The dry endotracheal tube & catheter sections were then mounted onto aluminium stubs, cool sputter coated with gold and imaged by scanning electron microscopy (SEM) using a Hitachi S3000H scanning electron microscope.

2.2.4 Proteomics- Cellular protein analysis by one dimensional SDS - PAGE

2.2.4.1 Protein extraction and fractionation

Bacterial cultures of planktonic and biofilm phases grown in serum SAPI, TSB or DMEM were harvested by centrifugation at 8000 x g for 10 minutes at 4°C, washed twice in 10mM Tris pH 7.5 or Tris EDTA buffer or PBS as appropriate, and the bacterial pellet re-suspended in 10mM Tris pH 7.5 or Tris EDTA buffer or PBS, as appropriate buffer for each test microorganism. The cell suspensions were frozen overnight at -80° C to increase the subsequent lysis of the bacterial cells by sonication. The samples were defrosted and were sonicated by a sonicator (Soniprep 150, Sanyo) which was set at 6-8 microns. The cell suspensions were sonicated in 15 second treatments followed by 45 second cooling in an ice water bath. The bacterial sonicates were then centrifuged at 10000 x g for 10 minutes to remove debris and unlysed cells; the supernatant contained total bacterial protein fraction (membrane and cytoplasmic proteins). Sample preparation and protein fraction will be explained in detail for each test bacteria in the respective chapters.

2.2.4.2 Preparation of proteins for SDS PAGE

The concentration of protein samples was measured by recording the absorbance at 280nm using a Nano Drop 1000 spectrophotometer (Thermo Scientific) according to the manufacturer's protocol. A 2 µl sample of each protein extract was placed on the Nanodrop and the absorbance at 280nm was measured. Nano pure water was used as a blank. Proteins samples were diluted or concentrated by freeze drying in a freeze dryer as required. Protein concentrations were normalised between tests and controls by addition of buffer as appropriate.

For analysis by SDS¬PAGE, the proteins were mixed with 4X sample loading buffer (at a 3:1 ratio). The proteins were denatured and solubilised by boiling at 95 °C for 5 minutes. The protein lysates were then centrifuged at 10,000 rpm for 1 minute in a microcentrifuge to remove debris and unlysed cells, and loaded directly onto SDS-PAGE gels.

2.2.4.3 Analysis of proteins by SDS-PAGE

Protein samples prepared as decribed in section 2.2.4.2 and prestained protein markers were loaded onto 12% mini (10 cm x 8 cm; 1 mm thick) SDS-PAGE gels. Proteins were separated using a Mini PROTEAN II system (BIORAD, UK) at a constant current (30mA/gel) for approximately one hour or more as per requirement. All gels were separated in 1X electrophoresis buffer. After electrophoresis, gels were stained in Coomassie stain and destained to visualise the protein bands. SDS PAGE gels were preserved in nanopure water at 4 °C until a picture was taken or sequenced.

Proteins profiles were obtained by scanning the gels using a GS-710 Densitometer connected to Quantity one software (BIO-RAD, UK) and analysed using Image J (NIH) software.

2.2.5 Data Analysis

All experiments were performed in duplicate or triplicate on at least 3 separate occasions. Graphs were plotted using Graph Pad Prism Program. Where appropriate, statistical analysis was carried out using One-way ANOVA (Analysis of Variance), two-way ANOVA with Dunnet's or Bonferroni post hoc tests using Graph Pad Prism Program. Statistical significance was indicated by a P value of less than 0·05.

All the microscopic and gel images were analysed using "Image J" version 1.46m software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland; http://rsb.info.nih.gov/ij/) image processing program.

Chapter Three

Phenotypic analysis of the effects of catecholamine inotropes on the growth, virulence and biofilm formation of *Pseudomonas aeruginosa*

3.1 Introduction

Pseudomonas aeruginosa causes serious infections in immunocompromised hosts, including patients with human immunodeficiency virus infections, cancer and those suffering severe burns (Pollack, 2000, Gaines *et al*., 2005). It is also the leading causative agent of chronic lung infections respiratory failure, and ultimately, death in cystic fibrosis (CF) patients (Baltch, 1994, Davis *et al*., 1996). *P. aeruginosa* is also the best-studied for microbial biofilm formation. Clinically, formation of these biofilms is an important issue in the pathogenesis of the bacterium in ventilator-associated pneumonia (VAP), urinary and peritoneal dialysis catheter infections, bacterial keratitis, otitis externa and burn wound infections (Driscoll *et al*., 2007) (see the description of *P.aeruginosa* in section 1.4.1 for more information).

Hospital associated pneumonia is a major threat to patients in intensive care units (ICU) who are receiving mechanical ventilation (CDC, 2004, Alp and Voss, 2006). Ventilator associated pneumonia is defined as nosocomial pneumonia occurring in a patient after 48 h of mechanical ventilation via an endotracheal or tracheostomy tube (Pneumatikos, *et al*., 2009). VAP is the most frequent nosocomial infection in the intensive care unit (ICU) occurring in 8–28% of mechanically ventilated patients which results in high mortality, morbidity and increase health-care costs (Cairns *et al*., 2011, Garau and Gomez, 2003, Alp and Voss, 2006). VAP is classified as early-onset when occurring within <96 hours of intubation and late-onset VAP if occurring ≥96 hours after intubation has begun, based on the time period between diagnosis of the pneumonia and initial hospital admission (Hedrick *et al*., 2008).

The predominant organisms responsible for human VAP infection are *S. aureus, P. aeruginosa, Acinetobacter baumanii* and members of Family *Enterobacteriaceae*. However, the precise causative agent depends on the population of patients in an ICU, duration of hospital stay, and prior antimicrobial therapy (Chastre and Fagon, 2002). Gram-negative bacilli are responsible for over 60 % of VAP cases with *P. aeruginosa* being responsible for half of these infections (Garau and Gomez, 2003). Risk factors for VAP are multiple and include the patient's body position, gastric-over distension, endotracheal tube intubation (ET), contamination of ventilator circuits, length of hospital stay, the presence of a nasogastric or an orogastric tube, male gender, age (over 60 years), the presence of acute respiratory distress syndrome, organ failure, coma, chronic obstructive pulmonary disease, tracheostomy, re-intubation, neurosurgery and cranial trauma (Alp and Voss, 2006, Augustyn, 2007).

Endotracheal intubation is the most important independent risk factor associated with VAP (Alp and Voss, 2006). The ET tube enables free passage of air to and from lungs and ventilates the lungs, and in critically ill patients tracheal intubation is performed in order to protect the airway from aspiration and facilitate the use of mechanical ventilation, (Pneumaticus *et al*., 2009). Endotracheal tubes are placed through the highly colonized oropharynx and larynx into the sterile tracheobronchial tree (Safdar *et al*., 2005), and its position disrupts the cough reflex. This ultimately promotes accumulation of tracheal and bronchial secretions and mucus, as well as direct access of inhaled particles to the airways, thus contributing to the pathogenesis of VAP (Pneumaticos, *et al*., 2009, Cairns *et al*., 2011). Previous work has shown that microorganisms may adhere to the ET and produce biofilms, making them relatively resistant to prophylaxic and curative antibiotics (Adair *et al*., 1999). It has been

suggested that dissemination of such microorganisms from the biofilm into the airways may occur upon insertion of a suction catheter, and fragments of ET biofilm may be uprooted and carried further into the lung by ventilator gas flow and contribute to the aetiology of VAP (Inglis, 1993, Inglis *et al*., 1995, Adair *et al*., 1999).

Although a number of risk factors for VAP have been identified such as duration of mechanical ventilation, tracheal intubation, VAP by bacteria such as *P. aeruginosa* still remains a major clinical problem (Morehead and Pinto, 2000, Alp and Voss, 2006). A risk factor that has not been addressed clinically is the consideration of whether medications prescribed to the intubated patient may influence their risk of infection. Previous work has shown that the stress hormones norepinephrine, epinephrine and dopamine and certain of their metabolites can all stimulate bacterial growth and virulence and biofilm stimulation (Lyte and Ernst, 1992, Freestone *et al*., 1999, 2002, 2008, Lyte *et al*., 1999, 2003). Catecholamines as inotropic drugs, particularly norepinephrine and dopamine, are prescribed for up to 50% of intensive care patients (Smythe *et al*., 1993). As well as the prescribed inotropes, ventilated patients are very sick and as a result both chronically and acutely stressed resulting in raised endogenous catecholamine levels (Schmidt and Kraft, 1995). Therefore, the study in this chapter was carried out based on the hypothesis that catecholamine inotropes might affect the growth and virulence of *P. aeruginosa*, contributing to its ability to cause VAP. The findings of this research described in this chapter has at the time of writing just been accepted for publication in the journal CHEST (Freestone *et al*. 2012).

Catecholamine inotrope doses

Thompson et al. (1999) showed that in some patients undergoing conventional cardiac surgery, when treated with 3µg/kg/min dopamine, norepinephrine plasma levels rose to 9.24 $\pm$ 2.98 µM. and epinephrine to 2.4 $\pm$ 2.51 µM (Table 3.1). Girbes et al. (2000) showed that in septic shock patients medicated with 6µg/kg/min dopamine, catecholamine plasma levels in some patients rose to greater than 5µM. These measurements were carried out using reverse phase high-pressure liquid-chromatography (HPLC) with electrochemical detection (Thompson *et al*., 1999).

Dopamine has a half life of several minutes and is infused at 1-15 µg/kg/min, and steady state levels in plasma vary according to infusion levels and general metabolic fitness, with critically ill patients showing slower clearance rates (Juste *et al*. 2004, Freestone *et al*., 2010). There is therefore a wide variation in dopamine plasma concentrations in patients receiving inotrope supplementation, ranging from ~50 nM (Johnston *et al*., 2004) to nearly 5,000 nM (5 µM) (Girbes *et al*., 2000).

Endotracheal administration of epinephrine has been recommended if intravenous access is contraindicated, and up to 2 mg of epinephrine may be directly applied via the ET (Raymondos *et al*., 2000). Epinephrine (as a 300 µM solution) is also occasionally directly nebulised through the ET to reduce airway inflammation (Stannard and O'Callaghan, 2002).

Table 3.1 Catecholamine inotrope dosage

The data shows mean plasma catecholamine concentrations in healthy people (Goldstein *et al*., 2011) and in patients during cardiac surgery (Thompson *et al*., 1999)

Inotrope	Healthy state	After anesthesia and before surgery	After surgery
Epinephrine	~0.2 nM	1.59 µM	2.67 µM
Norepinephrine	~1.0 nM	4.46 µM	9.24 µM

Aim

The aim of this study was to determine if exposure of *P. aeruginosa*, a major cause of VAP, to catecholamine inotropes commonly prescribed to ICU patients, at the dose levels prescribed in ICU patients affected its growth and capacity to form biofilms.

3.2 Specific Methods

3.2.1 Culture Conditions

P. aeruginosa strains, a Clinical Isolate (CI) (isolated from a Leicester Royal Infirmary pneumonia patient) and reference strain (UCBPP-PA14) were inoculated from overnight grown cultures grown in Luria broth at low cell density (approximately 50-100 CFU per ml) into serum SAPI supplemented in order to more closely approximate *in vivo* conditions and numbers of bacteria present at the beginning of an infection within a mammalian host (Freestone *et al*., 1999). Catecholamines levels were concentrations within the range of those reported in the plasma of dopamine medicated patients (Thompson *et al*., 1999; Girbes *et al*., 2000). A non-plasma concentration of 50 µM was also used to determine if higher doses of inotropes were equally, or more effective, as the clinical levels of the drugs. Also, ferric nitrate $Fe(NO_3)_3$ was used in

certain experiments to compare whether catecholamine inotrope effects were similar to that of iron.

Non- catecholamine inotropes such as vasopressin and phenylephrine were used to investigate whether these drugs were also equally effective with respect to stimulation of growth and biofilm formation. Different concentration ranges of vasopressin (0 – 0.001 units/ml) and phenylephrine (0 – 0.01 µg/ ml) were used in the analysis based on their clinical concentration (Tsuneyoshi *et al*., 2001, Russell *et al*., 2008).

3.2.2 Investigation of the mechanism by which catecholamines induce of *P. aeruginosa*

3.2.2.1 Binding of transferrin

To assess if binding of transferrin was occurring during the catecholamine growth induction process, the *P. aeruginosa* strains were grown overnight, harvested at 10,000 x g for 10 minutes, washed twice in PBS and the pellet was re-suspended in the binding assay medium. Binding assays were performed in duplicate 2 ml volumes of SAPI-medium containing 50mM Tris-HCl (SAPI-Tris), pH 7.5 to which 0.1 µg/ml of horse radish peroxidase-conjugated transferrin (HRP-Tf) was added. Cultures were incubated for one hour at 37°C in a 5% CO_2 humidified incubator, harvested by centrifugation at 5000 x *g* for 5 minutes, washed in PBS three times to remove any non-bound transferrin, and then re-suspended in 0.2 ml of SAPI-Tris. To comparatively visualise the Tf-binding, the washed CI and PA14 cell suspensions were serially diluted in 2-fold steps, and 10 µl was spotted onto nitrocellulose membrane. Visualisation of any HRP-Tf binding was achieved using enhanced chemiluminescence and autoradiography.

3.2.2.2 Bacterial uptake of Iron from Transferrin

a. ^{55}Fe labelling of Tf

[^{55}Fe]-Tf was prepared according to Freestone et al. (2000). Human Apo-Tf was incubated for 5 hours at 37 ºC in the presence of 25 µCi of $^{55}FeCl_3$ (1.5 µg of Fe / mg of protein), using 2 mM of sodium citrate as the iron donor. Unincorporated ^{55}Fe was removed by two rounds of spin column chromatography (Micro Bio-spin 6 columns, BIO-RAD, UK, 732-6221) equilibrated in 0.10 M Tris-HCl, pH 7.5. ^{55}Fe transferrin was added at a concentration of 1·5 mg L^{-1} (equivalent to 10^5 cpm of ^{55}Fe radiolabel) (Freestone *et al.*, 2000, 2002).

b. Intracellular ^{55}Fe[Tf] uptake

The *P. aeruginosa* cultures were inoculated at approximately 50-100 CFU per ml into serum-SAPI medium supplemented with 10^5 cpm of ^{55}Fe-transferrin along with no additions, and 5 µM and 50 µM norepinephrine. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator for 24 h, following which cells were harvested by centrifugation at 5000 x *g* for 5 minutes, washed in PBS, resuspended in 100 µl of PBS and assayed for cell numbers using plate analysis. ^{55}Fe incorporation into bacterial cells was measured after mixing the 100 µl suspensions with 2 mL Emulsifier safe scintillant (Canberra-Packard, Pangbourne, UK), followed by counting in the tritium channel of a Minaxi Tri-Carb 400 series scintillation counter (Canberra-Packard, USA) (Freestone *et al.*, 2000; 2002).

3.2.2.3 [^{3}H] NE binding assays

For analysis of catecholamine internalisation, cultures were grown in serum-SAPI containing 0.5 x 10^5 cpm per ml of ^{3}H-Norepinephrine with either no additions (control), 5 μM and 50 μM norepinephrine. Cells were analysed for growth and ^{3}H-NE internalisation was measured in scintillation counter as described above for the ^{55}Fe-transferrin assays (Freestone *et al.*, 2000). The growth levels (CFU/ml) of the cultures were determined by pour plate analysis.

3.2.2.4 Intracellular iron analysis

Measurements of *P.aeruginosa* intracellular iron levels were determined by extracting the total protein from control and inotrope-treated bacteria. The late exponential phase overnight cultures were washed and resuspended in 5 ml of serum SAPI medium supplemented with no additions (control) and 5 μM and 50 μM inotropes (Dopamine NE, Epi) and grown for 24 h in a humidified CO_2 incubator. Bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes and washed 3 times in 10 mM Tris-HCl, pH 7.5 to remove any Fe-containing serum proteins. The bacterial pellets were re-suspended in 500 μl of 10 mM Tris-HCl, pH 7.5 and sonicated (five 15 sec cycles) as described in section 2.4. The cell lysates were centrifuged twice for 10 minutes at 13,000 rpm in order to remove cellular debris and the supernatant frozen at -80°C and lyophilised. Total iron content of the protein samples was measured using inductively coupled plasma optical emission spectroscopy (ICPOES), courtesy of the University Leicester Geology Department.

3.2.3 Pyoverdine measurements

Production of the *P. aeruginosa* siderophore pyoverdine was measured using a fluorimetric assay (Adonizio *et al.*, 2008). After 48 h growth (to ensure control and test cultures reached similar culture densities) cell numbers were determined by pour plate analysis, bacteria were removed by centrifugation (10,000 x g for 10 minutes at 4°C) and the cell free supernatant filter-sterilised by passage through a 0.2 micron acrodisc syringe filter. Supernatants were diluted 1:10 with 10 mM Tris-HCl buffer, pH 7.5, and measured for pyoverdine using the fluorimetric function of a Varioskan spectrophotometer (Thermos, UK) set at 405 nm (excitation wavelength), and measuring the emission at 465 nm. Bacterial culture supernatants were corrected for any endogenous fluorescence due to serum proteins, and pyoverdine activity was measured in terms of relative fluorescent units (RFU) per normalised OD_{600}/ml of bacterial culture.

3.2.4 Twitching motility

Twitching motility assays were performed in LB medium solidified with 1.0% agar supplemented with various concentrations of catecholamines. Plates were dried overnight at room temperature, and *P. aeruginosa* strains were point inoculated at the bottom of the Petri plate. After 2 days, the twitching distance along the plastic–agar interface (at the bottom of the agar plate) was analysed both qualitatively & quantitatively. The agar was carefully removed, twitching zones attached to the petri plates were visualized by staining for 1 minute with 1% (wt/vol) crystal violet and their diameters measured (O'Toole and Kolter, 1998a, Patriquin *et al.*, 2008).

3.2.5 EPS assay

P. aeruginosa forms a pellicle-biofilm at the air/liquid interface of broth cultures which requires the involvement of the *pel* operon (Friedman and Kolter, 2004a,b). Pel-dependent EPS production was quantified as described previously (Ueda & Wood 2009; Lee *et al.*, 2007) based on the amount of Congo red that binds to the EPS. Reference strain PA14 and the clinical isolate cultures were grown overnight in serum SAPI supplemented with and without catecholamine inotropes (5 µM and 50 µM) at 37 °C in a humidified CO_2 incubator; 1 mL of each culture was washed and re-suspended in 500 µL of T-Broth. The bacterial suspensions in T-broth (500 µL) were then incubated with 40 µg/mL Congo-red at 37°C in a shaking incubator at 250 rpm. After 2 hrs, the bacteria and bound Congo red were collected by centrifugation and the amount of Congo red remaining in the supernatant was determined by measuring the absorbance of the supernatant at 490 nm using a spectrophotometer. T-broth which was supplemented with 40µg/mL Congo-red dye was used as the blank.

A fluorescence microscopy analysis of surface associated biofilm EPS stained with SYPRO ruby matrix stain after 48 h incubation was also carried out as described in section 2.2.3.1 and 2.2.3.2.

3.2.6 Total cellular protein analysis of planktonic and biofilm bacteria

Overnight optical density-normalised cultures of PA14 and CI were diluted to OD_{600} ~ 0.1. The diluted culture (0.5 ml) was then used to inoculate 50 ml of serum SAPI medium in polystyrene petri dishes (140mm diameter, triple vent, Sterilin, Newport, UK) to give a final population density of ca. 10^6 CFU/ml. Cultures were incubated with and without catecholamine inotropes at 5 and 50 µM concentrations (DO, NE, Epi); Fe

in the form of ferric nitrate (50 µM) was used as a positive control and the cultures were all incubated at 37° C in a 5% CO_2 incubator. After 48 h planktonic bacteria were removed, the plates were washed with PBS six times to remove the non-adherent bacteria and the biofilms were detached by scraping the petridish with a cell scraper. The biofilm cultures were harvested by centrifugation for 10 min at 16,300 x g at 4°C. The bacterial pellets were resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0), containing 0.3 mg of phenylmethylsulfonyl fluoride/ml, and cells were lysed by sonication (6 treatments for 10 s at 4 W, maintaining a temperature of 4°C). Cell debris and unbroken cells were removed by centrifugation at 30,600 x g, for 30 min at 4°C. Total protein concentrations were quantified, and the cell protein samples processed for SDS PAGE as described in section 2.2.4. Simultaneously, overnight optical density- adjusted cultures of PA14 and CI were diluted to OD_{600} 0.2, inoculated into serum SAPI incubated at 37° C in a 5% CO_2 incubator for 18 hrs. Planktonic cultures of both strains were harvested, washed and resuspended in TE buffer and processed for electrophoresis as described above for the biofilm bacteria (Sauer *et al*., 2002).

3.2.6.1 Protein Identification

Protein identification was carried out by the University of Leicester, in house Protein Nucleic Acid Chemistry Laboratory (PNACL), using the LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Briefly, gel plugs or lanes from SDS-PAGE gels were excised into equal slices and tryptic digests performed. The digested samples were then subjected to Liquid Chromatography - tandem mass spectrometry (LC/MS-MS).

The resulting mass spectrometry data were used to identify the proteins excised by peptide mass fingerprinting with tandem MS (MS/MS) and searched against Uniprot *Pseudomonas aeruginosa*_20100812 database. Scaffold software (version Scaffold_3.3.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al*., 2003).

3.3. Results

3.3.1 Catecholamine inotropic agents increase *P. aeruginosa* growth

Incubation of an inoculum of around 10^2 CFU per ml *P. aeruginosa* strains CI and PA14 in serum-SAPI for 18 hrs with the inotropes (NE, dop, Epi (at 5 µM and 50 µM) resulted in an up to a significant 50 – 100 -fold increase in bacterial numbers over the un-supplemented control cultures ($P<0.05$ for both strains) (Figure 3.1). At 50 µM concentration the inotropes had a similar effect on growth of both strains as 5µM Iron. Longer incubations (48 hrs) showed control and inotrope cultures had reached a similar cell density of around 10^9 CFU/ml (data not shown).

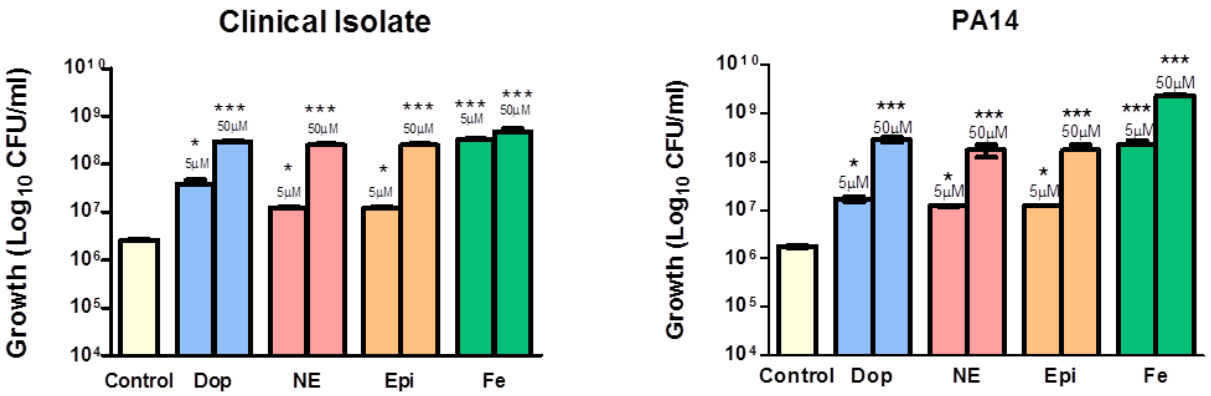


Figure 3.1 Effect of catecholamines on growth of *P. aeruginosa.*

Overnight cultures (Clinical Isolate and PA14) were diluted to about 100 CFU/ml into serum-SAPI with or without the catecholamines shown and Fe (all at 5 and 50 µM); the cultures were grown for 18h statically at 37^{o}C. Bacterial growth levels were enumerated by serial dilution of the cultures followed by plating onto LA. Values represent the means and standard deviations of triplicate platings from triplicate cultures. Keys: Dopamine (Dop), Norepinephrine (NE), Epinephrine (Epi). The symbols * indicate statistical significance of * P<0.05, ** P<0.01; *** P<0.001.

3.3.2 Growth induction mechanism

Iron limitation by the iron-sequestering protein transferrin is primarily responsible for the innate bacteriostatic nature of serum and blood (Ratledge and Dover, 2000). An investigation was therefore done to determine if the mechanism by which inotropes were inducing *P. aeruginosa* growth was via enabling bacterial access to transferrin-iron.

3.3.2.1 Transferrin binding assay

The transferrin binding assay images shown in Figure 3.2 confirmed that transferrin was directly bound by both *P. aeruginosa* strains during growth in serum-SAPI. A two fold dilution comparison was done to compare the binding abilities of both strains. The Clinical isolate strain's transferrin binding ability was comparatively higher than the reference strain PA14, since clear binding was observed even at very low inoculum densities.

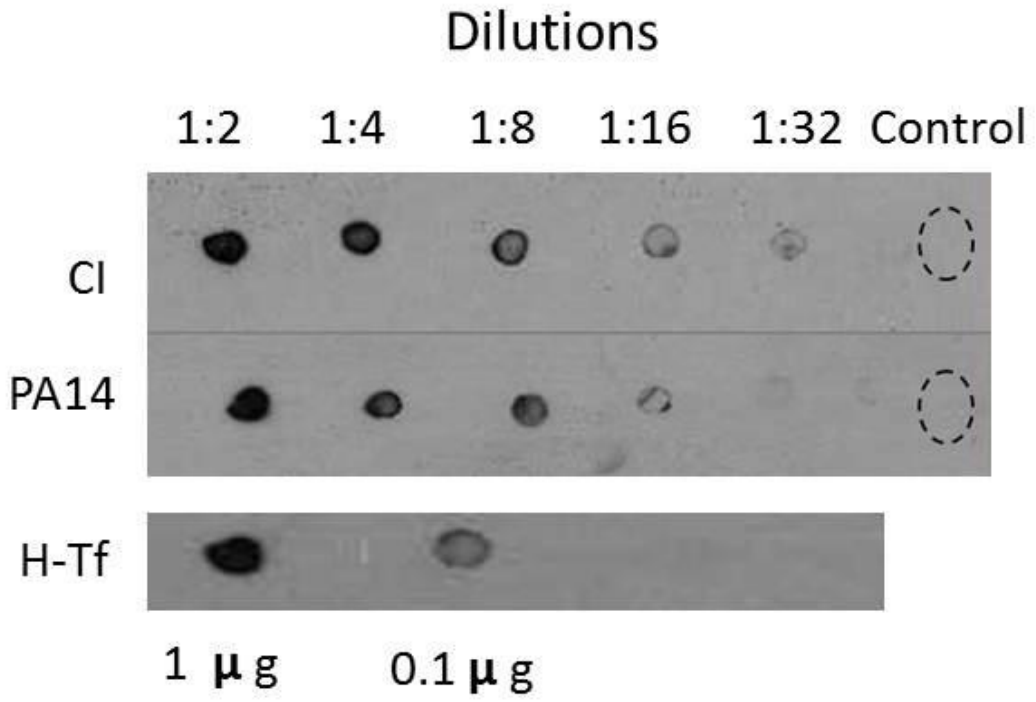


Figure 3.2. Transferrin binding by the Clinical Isolate (CI) and PA14 *P. aeruginosa* strains.

The upper 2 panels show transferrin binding blots of a series of dilutions of the CI and PA14 cultures (initial cell density around 2 x10^8 CFU/ml); the control consisted of similar numbers of bacteria to the 1:2 dilution but incubated without transferrin; the activity of 1.0 and 0.1 µg horse radish peroxidase-transferrin are shown in the lower panel.

3.3.2.2 Catecholamine inotropes facilitate Iron uptake from transferrin

To determine if the catecholamines delivering transferrin iron was part of the growth induction mechanism, the bacteria were grown in the presence of transferrin labelled with ^{55}Fe as described in section 3.2.2. It can be seen that the presence of NE (Figure 3.3) enabled significant increase in internalisation of the transferrin-^{55}Fe ($P<0.05$) at low concentration. At a higher concentration of NE the transferrin-^{55}Fe uptake by both of the strains was also significantly increased ($P>0.05$). This finding suggests that catecholamine inotropes are enhancing transferrin iron uptake by *P.aeruginosa*.

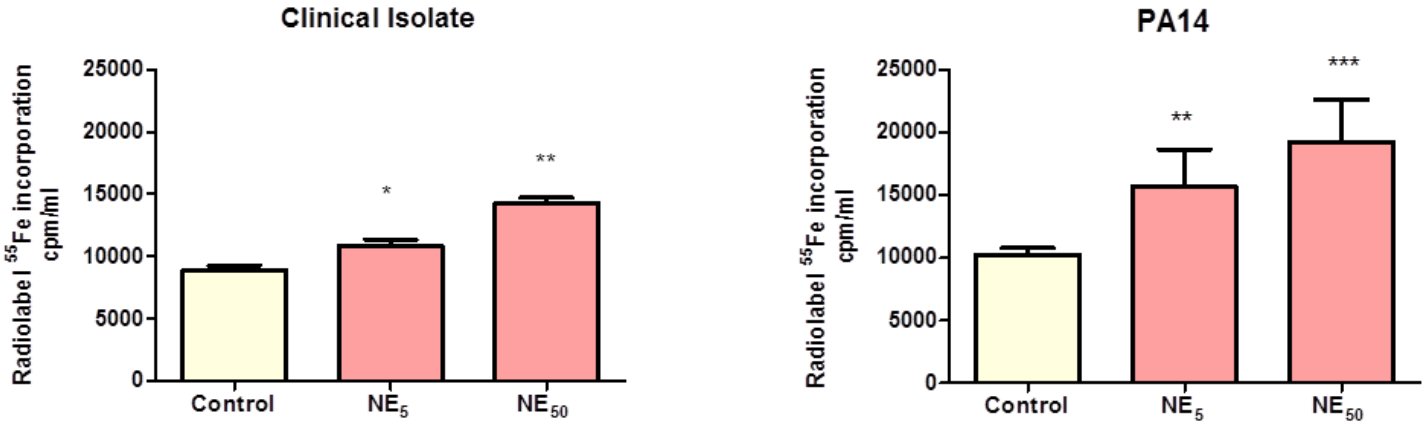


Figure 3.3 Iron uptake from ^{55}FeTransferrin by *P.aeruginosa* Clinical Isolate & PA14 strains

After normalising cell densities bacterial internalisations of iron of the control and in the presence of norepinephrine (NE) at 5µM and 50µM concentration (n=4). Values represent the means and standard deviations of representative bacterial internalisations of iron (in the form ^{55}Fe from ^{55}Fe-transferrin). The symbols * indicate statistical significance of * P<0.05, ** P<0.01; *** P<0.001.

3.3.3.3 ^{3}H-norepinephrine internalisation

The inclusion of tritiated norepinephrine into the *P. aeruginosa* serum-SAPI culture medium showed that during the growth induction process the inotrope was also internalised by the bacteria. Internalisation of ^{3}H-norepinephrine was significantly high when 5 or 50 μM unlabelled norepinephrine was present ($P<0.05$) (Figure 3.4). Even after longer incubation (48 hrs) and despite equivalence of cell numbers, repeat of the assays shown in Figures 3.3 and 3.4 revealed that the inotrope-treated bacteria still retained higher levels of transferrin-associated ^{55}Fe and higher levels of internalised ^{3}H-norepinephrine than control cultures (data not shown).

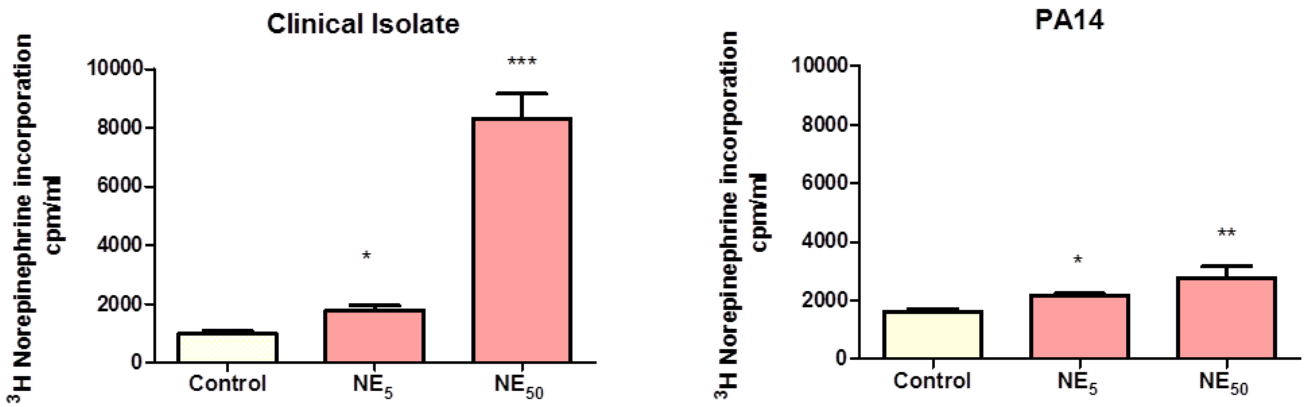


Figure 3.4 Norepinephrine internalisation by *P. aeruginosa* Clinical Isolate & PA14 strains.

Bars are representative bacterial internalisations of tritiated norepinephrine. Values represent the means and standard deviations of representative bacterial internalisations of tritiated norepinephrine (NE) from triplicate cultures. Keys: Norepinephrine (NE). The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$; *** $P<0.001$.

3.3.3.4 Iron levels in inotrope treated bacteria and proteomic analysis

In order to examine whether the bacteria grown in the presence of inotropes were iron replete, the intracellular iron levels in the total protein extracts of *P.aeruginosa* strains grown in serum-SAPI in presence or absence of inotropes and iron were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The ICPOES analysis of internal iron levels show that after normalising cell culture densities between control and catecholamine cultures, during growth the inotrope increased bacterial internalisation of Fe (Figure 3.5). Inotrope-treated bacteria all internalised significantly more Fe than controls ($P<0.05$). However, the clinical isolate showed a significant increase in iron internalisation only at the higher inotrope concentration ($P<0.05$). PA14 reference strain showed significant iron internalisation at both inotrope concentrations ($P<0.05$). However, internal Fe levels in the presence of free iron was higher for both strains than the inotropes. This finding is consistent with the ^{55}Fe-Tf iron uptake analysis in section 3.3.3.2.

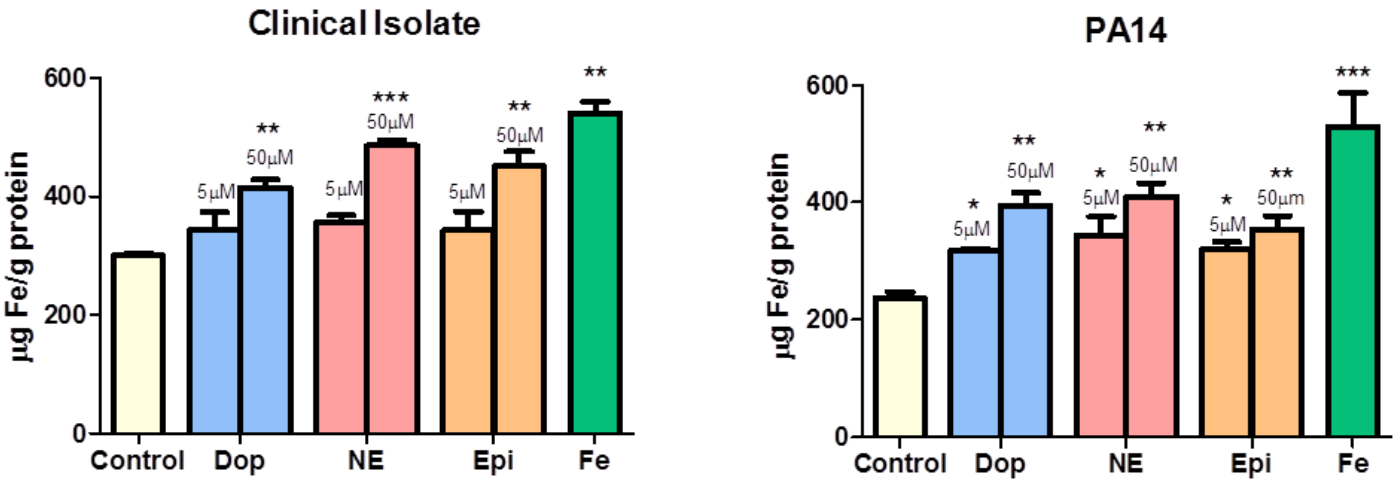


Figure 3.5 Analysis of the internal Fe levels in inotrope-treated *P. aeruginosa* Clinical Isolate & PA14 strains.

Bars are representative bacterial internalisations of iron (Fe). Values represent the means and standard deviations of representative bacterial internalisations of iron (Fe) from triplicate cultures. Keys: Dopamine (Dop), Norepinephrine (NE), Epinephrine (Epi). The symbols * indicate statistical significance of * P<0.05, ** P<0.01; *** P<0.001.

3.3.3 Inotropes increase Pyoverdine synthesis

Pyoverdine is an important *P. aeruginosa* virulence factor and a main ferric iron sequestering siderophore (Xiao and Kisaalita, 1998). Often, it is observed as a yellow-green pigment in culture supernatants. By carrying out a fluorimetric assay for pyoverdine on the culture supernatants of serum-SAPI-inotrope grown *P. aeruginosa* (n=4) it was found that the presence of the inotropes induced both *P. aeruginosa* strains to synthesise significantly more of the siderophore than control cultures ($P<0.001$) (Figure 3.6). At both 5 µM and 50 µM concentrations dopamine induced pyoverdin production was more than the other inotropes for both strains.

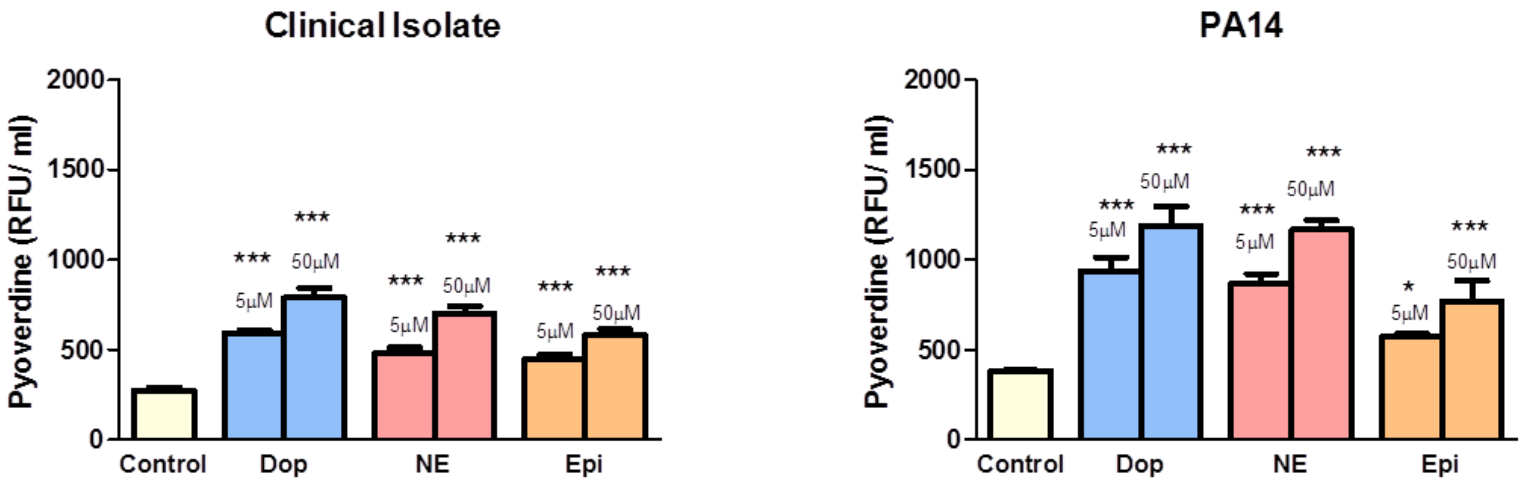


Figure 3.6 Pyoverdine levels of inotrope-treated CI and PA14 cultures.

The initial inoculum for strains CI and PA14 (n=4) was less than 100 CFU/ml; bacteria were grown for 48 hrs and assayed as described in Materials and Methods section 3.2.3. Keys: Dopamine (Dop), Norepinephrine (NE), Epinephrine (Epi). The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$; *** $P<0.001$.

3.3.4 Effects of catecholamine inotropes on biofilm formation

3.3.4.1 Biofilm formation assay

Biofilm development of the *P. aeruginosa* strains were studied by crystal violet microtitre plate biofilm formation assay. Biofilm formation on polystyrene of both the strains and in response to catecholamine in serum SAPI medium are shown in Figure 3.7. It can be seen that both 5 and 50 µM concentrations of the inotropes biofilm formation of the two *P. aeruginosa* strains. The biofilm formation assay incubation period was empirically determined by checking the biofilm formation at different time points (data not shown). The laboratory reference strain showed differences in biofilm formation with the addition of inotropes within 4 hrs (data not shown). However, the clinical isolate biofilm formation was slower and was significantly enhanced with the addition of catecholamines (dopamine, norepinephrine, epinephrine) in ~48 hrs ($P<0.05$). Since, the clinical isolate is much more relevant to this study than a much passaged reference strain, the biofilm formation assay results of 48 hrs incubation was used. Interestingly, the stimulatory effect of inotropes at 50µM on biofilm formation was equivalent to that of 5 µM free iron (Figure 3.7).

Microscope investigation of 48 hrs old cultures confirmed that for both strains the catecholamine inotrope treated clinical isolate and PA14 cultures showed more biofilm formation with more microcolonies than the unsupplemented controls, which, did not develop large microcolonies but instead produced instead small colony aggregates (Figure 3.8). Furthermore, at a higher concentration (50 µM) of the three inotropes, both strains showed even more dense and tightly packed microcolonies (Figure 3.8).

This suggests that in addition to enhancing growth the inotropes were also increasing *P. aeruginosa* biofilm development.

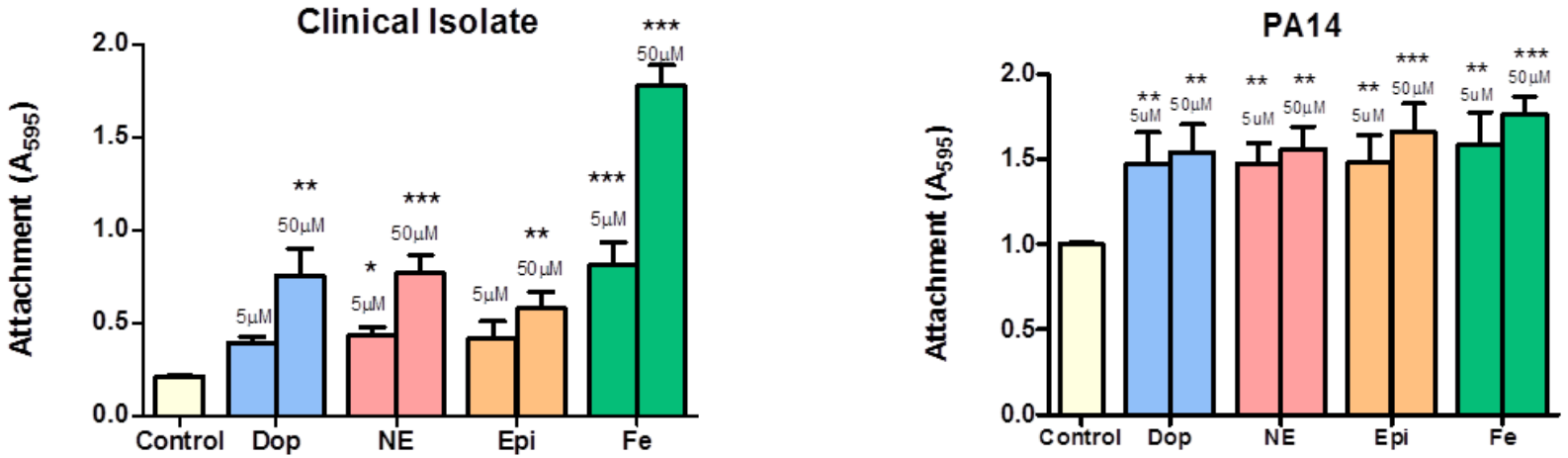


Figure 3.7 Inotrope and Fe effects on biofilm formation of strains CI & PA14 on a polystyrene surface.

Overnight *P. aeruginosa* strains CI and PA14 cultures were diluted 1:100 into serum-SAPI medium, and biofilm formation measured after 48hrs incubation. Data shown represent means and SD of 4 biological replicates from triplicate assays. Key: Dopamine (Dop), Norepinephrine (NE), Epinephrine (Epi). The symbols * indicate statistical significance of * P<0.05, ** P<0.01; *** P<0.001.

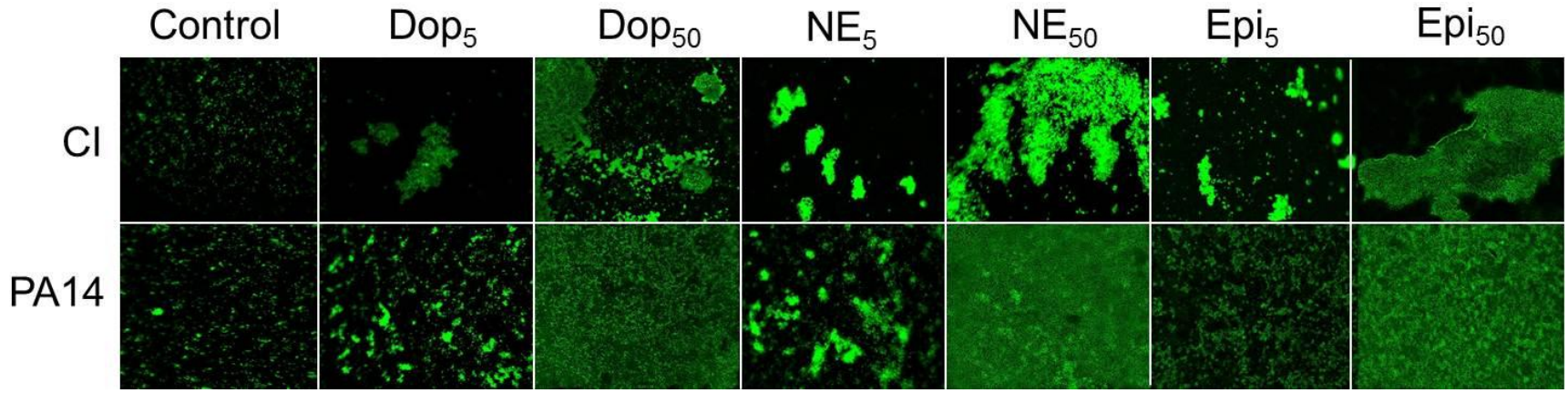


Figure 3.8 Catecholamine inotrope biofilm enhancement.

The panel of images show fluorescence micrographs of inotrope-enhancement of *P. aeruginosa* biofilm formation on polystyrene under 40 x objective of Nikon Eclipse Ti 2000 fluorescent microscope (Scale = 10 µm). *P. aeruginosa* PA14 strain and Clinical Isolate were inoculated at 10^6 CFU/ml onto polystyrene 96 well microtitre plate in the absence or presence of 5 µM and 50 µM norepinephrine, epinephrine and dopamine and incubated in serum-SAPI medium for 48 hrs, stained with live-dead biofilm stain (n=3). Keys: Dop-Dopamine, NE- Norepinephrine, Epi- Epinephrine.

3.3.4.2 Catecholamine inotropes enhance *P. aeruginosa* biofilm formation on endotracheal tubes

The presence of the endotracheal tube (ET) is thought to be a significant factor in both the initiation and development of *Pseudomonas* associated pneumonia (Adair *et al*., 1993, 1999). Figures 3.7 & 3.8 showed that in addition to enhancing growth, the inotropes might also increase *P. aeruginosa* biofilm formation. This investigation was then done in a more clinically relevant context (the endotracheal tube) to study the possibility of inotrope effects on endotracheal tube biofilm formation. Figure 3.9 shows the SEM of *P.aeruginosa* strain CI and PA14 incubated for 48 hrs in the presence of 5 µM norepinephrine, dopamine and epinephrine. It can be seen that compared to the un-supplemented control cultures, the inotropes induced extensive bacterial attachment and formation of biofilm (n=4). Preliminary time courses of growth of *P. aeruginosa* in serum-based media revealed that the rate of increase in bacterial numbers is bacterial density dependent (data not shown), and that at the inoculum used in the experiments culture (10^5 CFU/ml), the control and inotrope-treated cultures always showed similar rates of growth and final cell densities. This suggests that the catecholamine biofilm formation on the ET tube surface was not due to a simple direct growth-related effect, and that induction of the expression of genes involved in biofilm formation must have occurred. At a higher concentration (50 µM) inotrope stimulated even more copious biofilm formation (data not shown).

As shown in Figure 3.10 the inotropes induced *P. aeruginosa* biofilm architecture on the endotracheal tube. These figures reveal fragments of norepinephrine and dopamine induced biofilms that have become detached from the endotracheal tube. These SEM images of *P. aeruginosa* biofilm fragment different views with inotrope treatment are

shown in the figure 3.10. The underside showed flatter and more uniform in appearance biofilm and the upper side shows mushroom-like projections The full fragment image reveals the thickness of the biofilm is variable; however, the side edge view gives an indication of how dense the biofilm may have been prior to the SEM processing. This result indicates that the inotrope induced biofilm maturation.

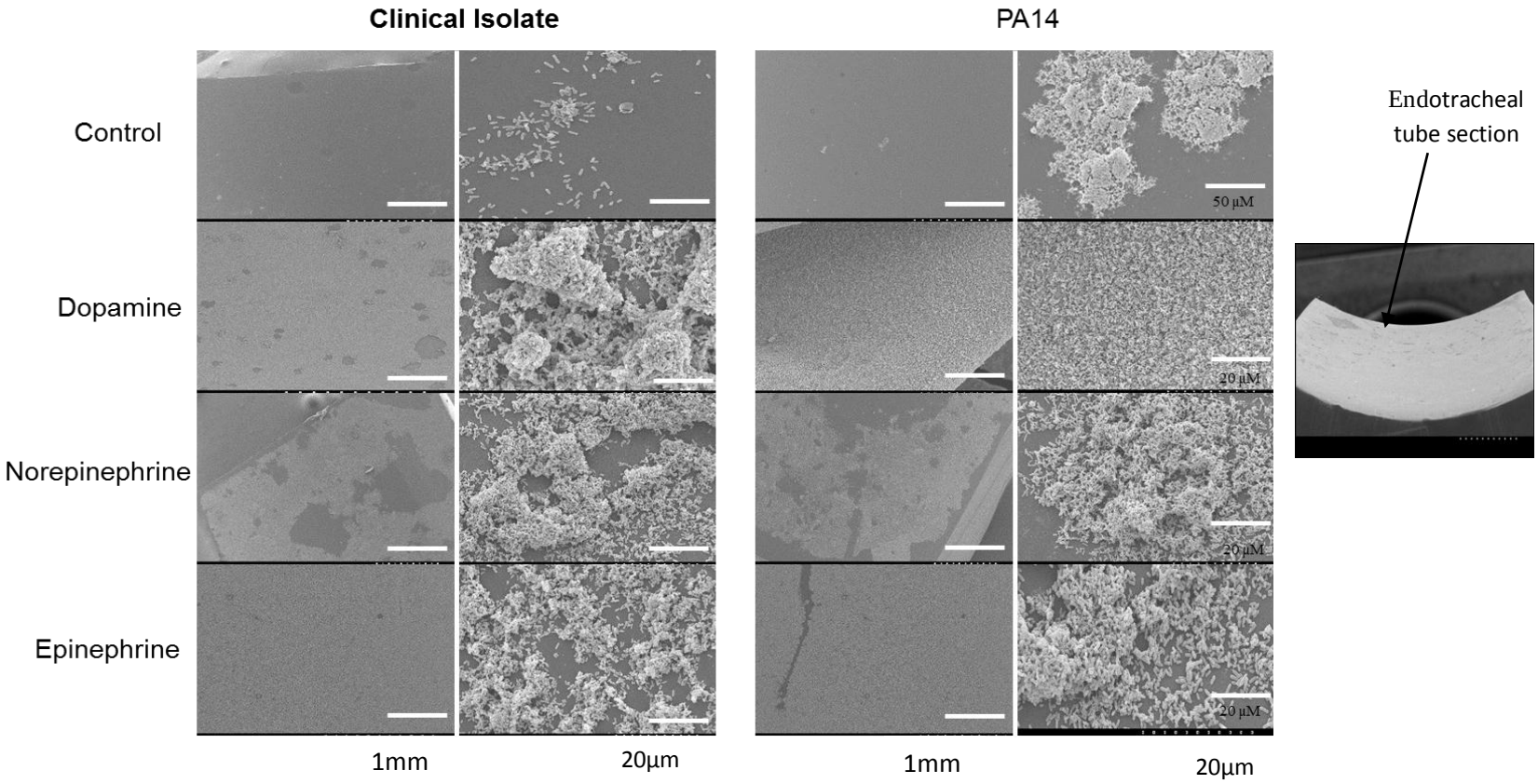


Figure 3.9 Scanning electron microscopic (SEM) images of inotrope-induced increases in *P. aeruginosa* biofilm formation on ET.

The panel of images show SEMs of catecholamine inotrope-enhancement of *P. aeruginosa* biofilm formation on endotracheal tubing. *P. aeruginosa* strain Clinical Isolate and PA14 were inoculated at $8x10^5$ CFU/ml onto sterile endotracheal tube sections in the absence or presence of norepinephrine (5µM), epinephrine (5µM) and dopamine (5µM) in serum-SAPI medium and incubated for 48 hrs as described in Materials and Methods 2.2.3.4 (n=3). The 4 sets of panels show representative SEMs (of 3 separate experimental sets) of the ETs at increasing magnification; the scales for each image are shown at the bottom of the figure.

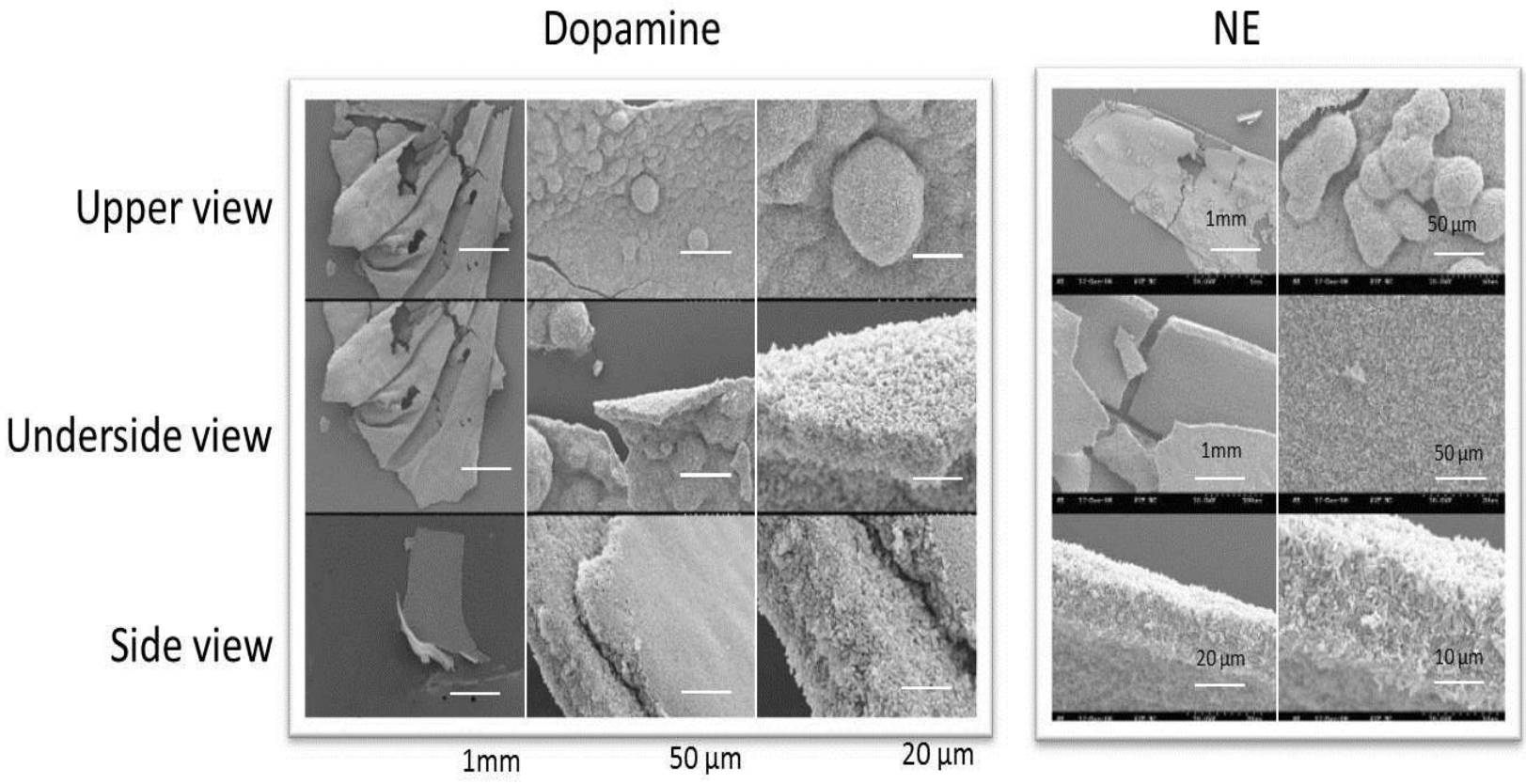


Figure 3.10 Architecture of inotrope-induced *P. aeruginosa* biofilms

The panels show scanning electron micrographs of the upper, lower and side architectural views of a similarly prepared biofilm fragment of Clinical Isolate formed in the presence of dopamine (5 µM) and NE (5 µM). The scales shown are for the bars on the scanning electron micrographs. Similar types of biofilm structure were also seen with epinephrine-treatment (data not shown).

3.3.5 Effect of catecholamine inotropes on *P. aeruginosa* motility

Biofilm development begins with the reversible and irreversible attachment to a surface. Motility plays an important role in *P. aeruginos*a biofilm formation (e.g. O'Toole & Kolter, 1998, Singh *et al*., 2002, Klausen *et al*., 2003, Singh, 2004, Patriquin *et al*., 2008). Therefore, a comparative analysis was made on any changes in motility of *P.aeruginosa* occurring in response to the catecholamine treatment of norepinephrine, dopamine and epinephrine.

3.3.5.1 Catecholamines and swimming motility

Inclusion of norepinephrine at either 5 µM or 50 µM significantly enhanced the swimming motility of both PA14 & CI (Figure 3.11) ($P<0.05$) for both strains. Although at 5µM, epinephrine and dopamine had a more moderate effect on motility did not reach statistical significance. However, the drugs significantly enhanced swimming motility of the bacteria at the higher concentration ($P<0.05$).

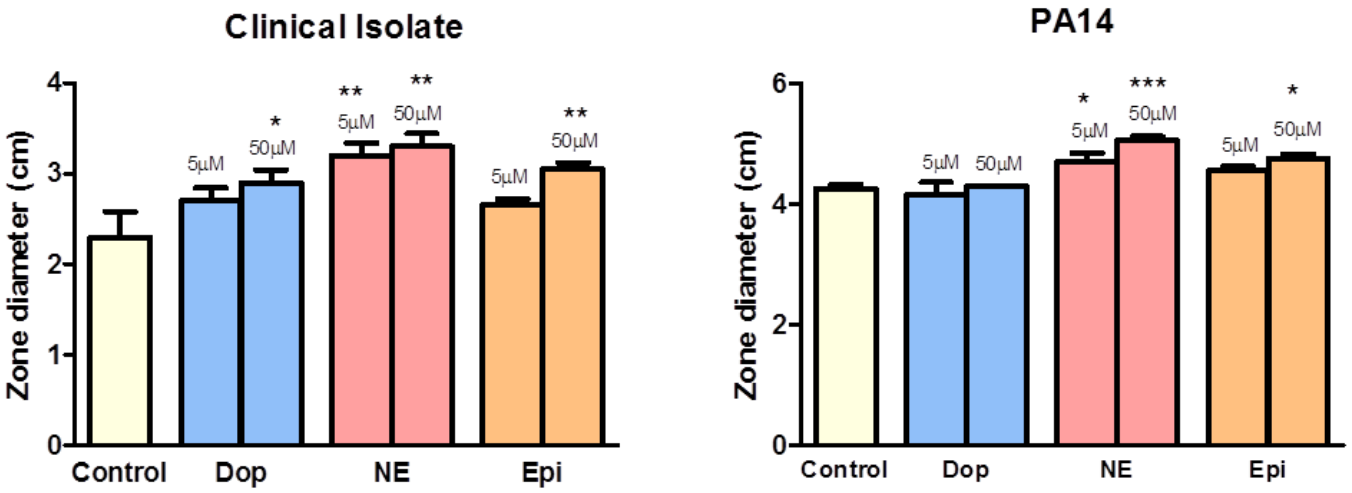


Figure 3.11 Effects of catecholamines on swimming motility of strains CI & PA14

In the presence of no inotrope (control) or 5 µM and 50 µM dopamine, norepinephrine or epinephrine (n=4). Keys: Dop-Dopamine, NE- Norepinephrine, Epi-Epinephrine. The symbols * indicate statistical significance of * P<0.05, ** P<0.01; *** P<0.001.

3.3.5.2 Catecholamines and twitching motility

Twitching motility is a surface translocation movement achieved by Type IV pili (fimbriae) present on the cell surface of *P. aeruginosa* (Whitechurch *et al*., 1990, O'Toole and Kolter, 1998a). In addition to forming a colony on the surface of the agar plate (1.0% agar), strains capable of twitching also form a haze of growth (the twitch zone) which surrounds the point of inoculation with a spreading colony morphology (O'Toole & Kolter, 1998 a, b, Whitchurch *et al*., 1990). Effects of inotropes on twitching motility were investigated, and Figure 3.12 shows that there were differences in twitching motility between the control and catecholamine inotrope-treated cultures of the CI strain. Dopamine, norepinephrine and epinephrine all enhanced twitching at both 5 and 50 µM. In contrast, PA14 twitching motility was less than that of the CI strain, and the catecholamine inotropes apparently did not have any effect. Iron had little effect on motility compared to the catecholamines (Figure 3.12 A). The twitching zone diameters of the inotrope treated CI cultures were significantly greater than the untreated control ($P<0.05$) except for dopamine at 5 µM concentration A reduction in twithching motility with epinephrine was observed and this did not have statistical significance (Figure 3.12 B). The PA14 strain did not show much difference in the twitching zone measurements except for a slight significant enhancement ($P<0.05$) with norepinephrine treatment at 50 µM concentration (Figure 3.12 B). This finding shows that the inotropes increase twitching motility of the clinical isolate, which thus may have contributed to the formation of the bigger *P. aeruginosa* microcolonies shown in Figure 3.8.

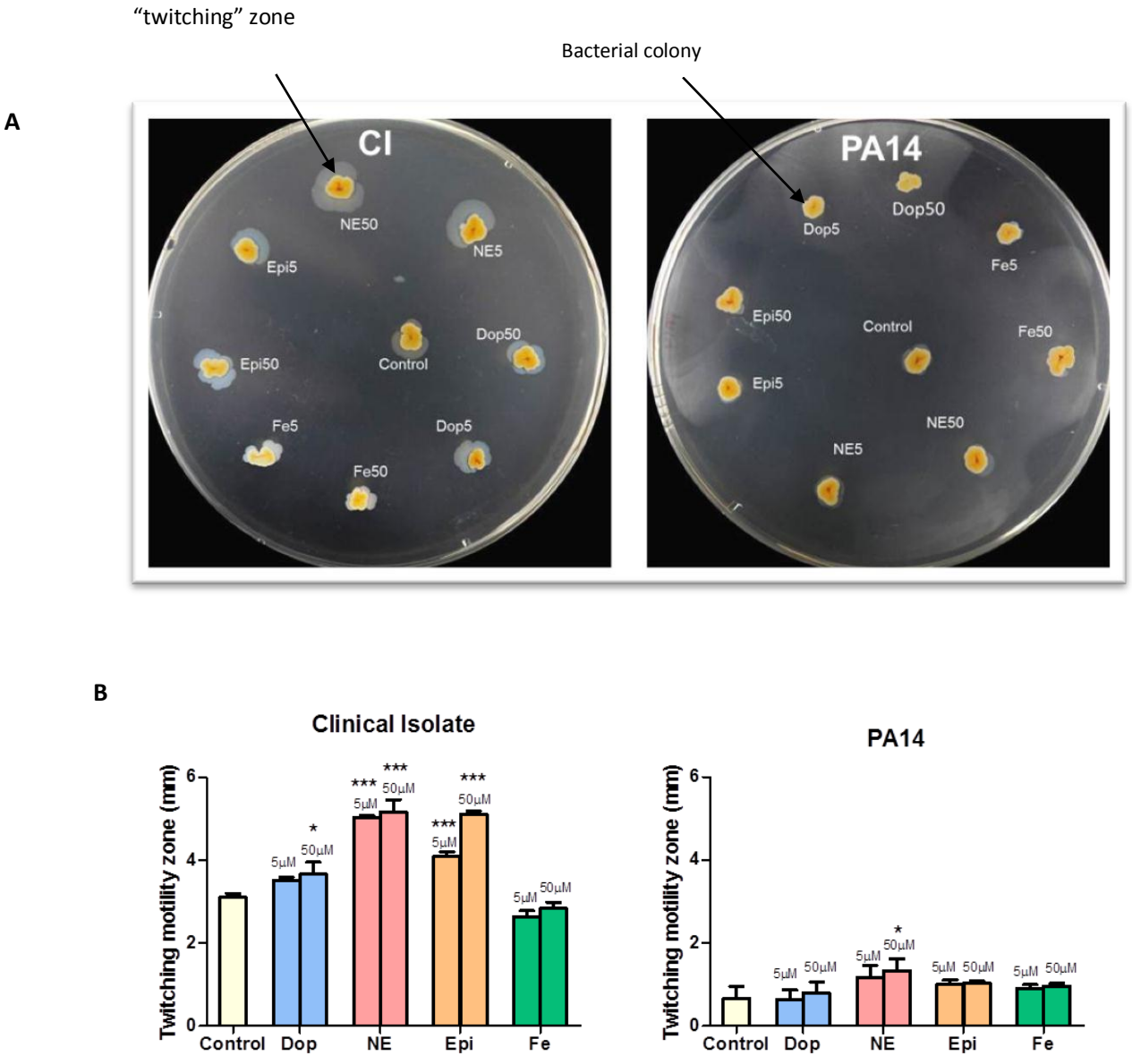


Figure 3.12 Twitching motility of *P. aeruginosa* strains CI and PA14

A.Twitching motility is shown in the absence and presence of Norepinephrine (NE), Dopamine (Dop), Epinephrine (Epi) & Fe and (5µM and 50µM) on 1.5% LB Agar. Representative images are shown of three separate experiments. B. twitching zones (diameter) measured after 48 h of incubation at 37°C. Error bars represent the standard deviation of the means for a representative assay performed in triplicate (n=3). Keys: Dop-Dopamine, NE- Norepinephrine, Epi-Epinephrine. The symbols * indicate statistical significance of * P<0.05, *** P<0.001.

3.3.6 Effect of catecholamine inotropes on biofilm EPS production

In *P. aeruginosa* PA14, the glucose-rich extracellular polysaccharide (EPS) of the biofilm matrix is formed by proteins encoded by the pel operon; Pel was shown to enable *P. aeruginosa* to form pellicle-biofilm at the air/liquid interface of broth cultures (Friedman and Kolter, 2004 a,b). The Pel-dependant EPS production at air/ liquid interface was investigated for both CI and PA14 as described in section 3.2.5. As shown in Figure 3.13, both strains did not show significant differences in the production of Pel EPS in the presence of low concentrations of the inotropes. Both strains formed pellicle after 24 hrs incubation.

The CI strain showed enhanced Pel polysaccharide production for epinephrine (5 and 50 μM) and dopamine at 50 μM concentration which was not significant ($p>0.05$). However, norepinephrine showed a reduced production compared to the control. The reference strain in PA14 showed increased pel polysaccharide production at both concentrations of all three inotropes tested except for norepinephrine which showed less production at 5 μM. Taken together with the pel-dependent EPS production data, the catecholamine inotropes slightly enhanced or reduced EPS production which which did not reach statistical significance ($P>0.05$).

Furthermore, fluorescence microscopic analysis of the 48 hrs biofilm matrix on polystyrene, showed differences in EPS production for both strains (Figure 3.14 A,B). The fluorescent intensity of whole well coverage of biofilm EPS quantified and the reference strain PA14 showed significant increase in the production of EPS with exposure to inotropes ($P<0.05$). The clinical strain showed a significant increase in EPS in the presence of 50 μM norepinephrine and epinephrine ($P<0.05$) and did not

reach significance at 5 µM concentration of three inotropes tested (P>0.05) (Figure 3.14 B). These results suggest that catecholamine inotropes do have an influence in the EPS production of *P.aeruginosa*.

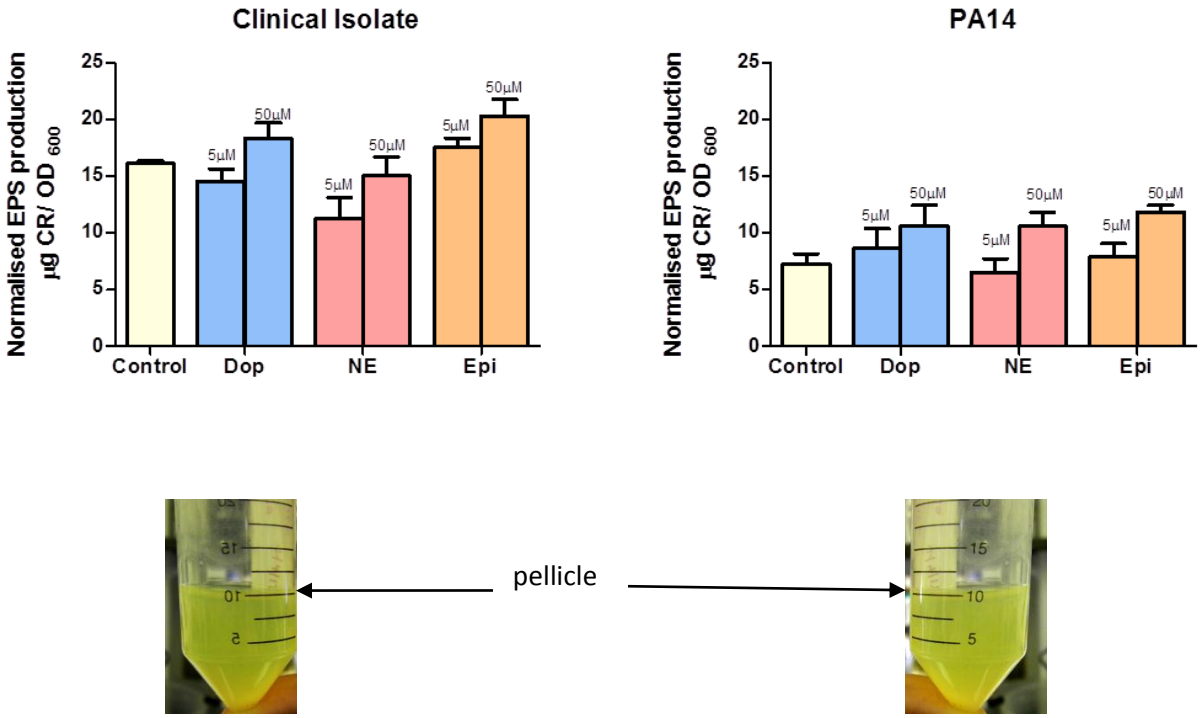


Figure 3.13 Effects of catecholamines on Pel polysaccharide dependant EPS production of strains CI & PA14

in the presence of no inotrope (control) or 5 and 50 µM dopamine, norepinephrine or epinephrine. Data shown represent means and SD of 3 biological replicates from triplicate assays. Keys: Dop-Dopamine, NE-Norepinephrine, Epi- Epinephrine. Images below the histograms are cultures showing thin pellicle formation at air- liquid interface showed no signficant differences (P>0.05).

A.

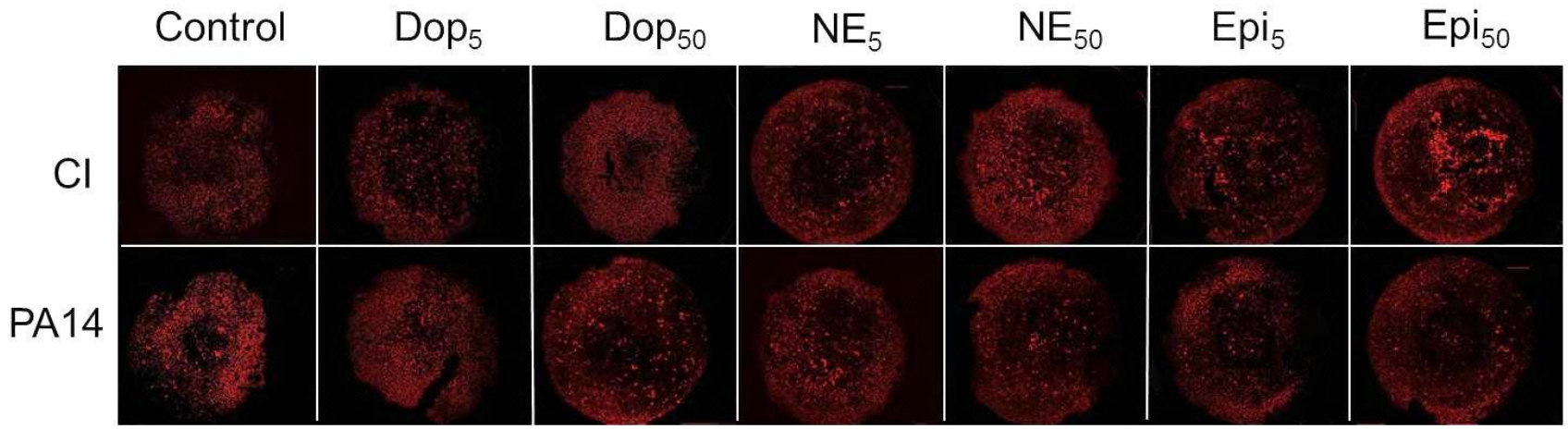


Figure 3.14 A Catecholamine inotrope effect on biofilm EPS. A. The panel of images show micrographs of inotrope effect on *P. aeruginosa* biofilm matrix on polystyrene. *P. aeruginosa* PA14 strain and Clinical Isolate were inoculated at 10^6 CFU/ml onto 96 microtitre well platesin the absence or presence of 5 and 50 µM inotropes and incubated in serum-SAPI medium for 48 hrs (n=3) and stained with SYPRO Ruby matrix stain and observed under 10x as described in section 2.3.2. Keys: Dop-Dopamine, NE- Norepinephrine, Epi- Epinephrine.

B.

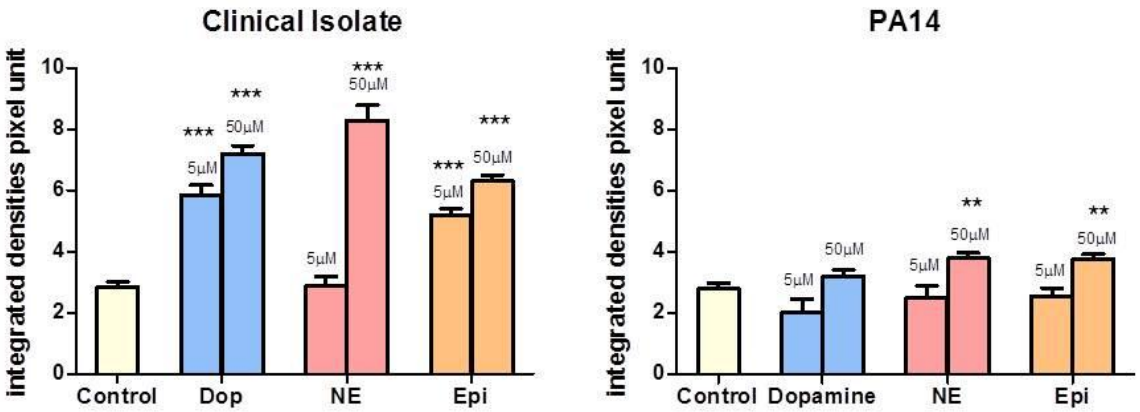


Figure 3.14 B Catecholamine inotrope effect on biofilm EPS (Quantification). Graph showing fluorescence intensities measured as integrated densities of the stained biofilm matrix. *P. aeruginosa* PA14 strain and Clinical Isolate were inoculated at 10^6 CFU/ml onto 96 microtitre well plates in the absence or presence of 5 µM and 50 µM inotropes and incubated in serum-SAPI medium for 48 hrs (n=3) and stained with SYPRO Ruby matrix stain and observed under 10 x as described in section 2.3.2. Keys: Dop-Dopamine, NE- Norepinephrine, Epi- Epinephrine. The symbols * indicate statistical significance of ** P<0.01; *** P<0.001.

3.3.7 Proteomic analysis of inotrope treated *P. aeruginosa*

The whole cell protein profiles of planktonic and biofilm bacteria of both strains were extracted and run on SDS-PAGE gels as described in sections 3.2.6. The 48 hrs biofilm proteins showed very similar profiles with and without the inotrope treatment catecholamines. However, there was a slight difference in protein expression in the size range of 50 - 70 KDa proteins with the treatment of 50 µM inotropes (3.16). The planktonic protein profiles of both strains are shown in Figure 3.15 and the expression patterns were compared and discussed in relation to free iron (section 3.3.3)

The whole cell protein profiles of untreated and inotrope and Fe stimulated bacteria grown in serum-SAPI are shown in figure 3.15. For both *P. aeruginosa* strains the protein profile grown in the presence of inotropes is more similar to the untreated control, compared to the Fe-grown bacteria. However, at 50 µM inotrope concentrations the bacterial protein profiles were more similar to those grown in excess iron. Protein identification of selected cell proteins of clinical isolate control and inotrope treated bacteria showed the presence of Ferric uptake regulator (Fur) only in the presence of inotrope (norepinephrine) (Table. 3.2). This finding further confirms that the inotropes are involved in iron acquisition.

The protein bands of interest which showed differences in expression had on sequence analysis a mixture of proteins and identification of these proteins separately was not easy and economical. In terms of the analysis, selected proteins showing differences in expression from the total cell protein lanes of the clinical isolate planktonic and biofilm profiles were excised and subjected to liquid chromatography mass spectrometry

(LC/MS-MS) analysis as described and identified based on Mascot score (described in section 3.2.6 & 3.2.7). The protein sequence data are attached in Appendix II.

The important planktonic and biofilm proteins which were found only in the presence of inotrope treatment are summarised in Table 3.2 (full data on the sequence results are shown in Appendix 1). Those proteins showing changes in expression were mainly involved in iron regulation, motility, protein synthesis, DNA binding, alginate synthesis and other metabolic process (Table 3.2).

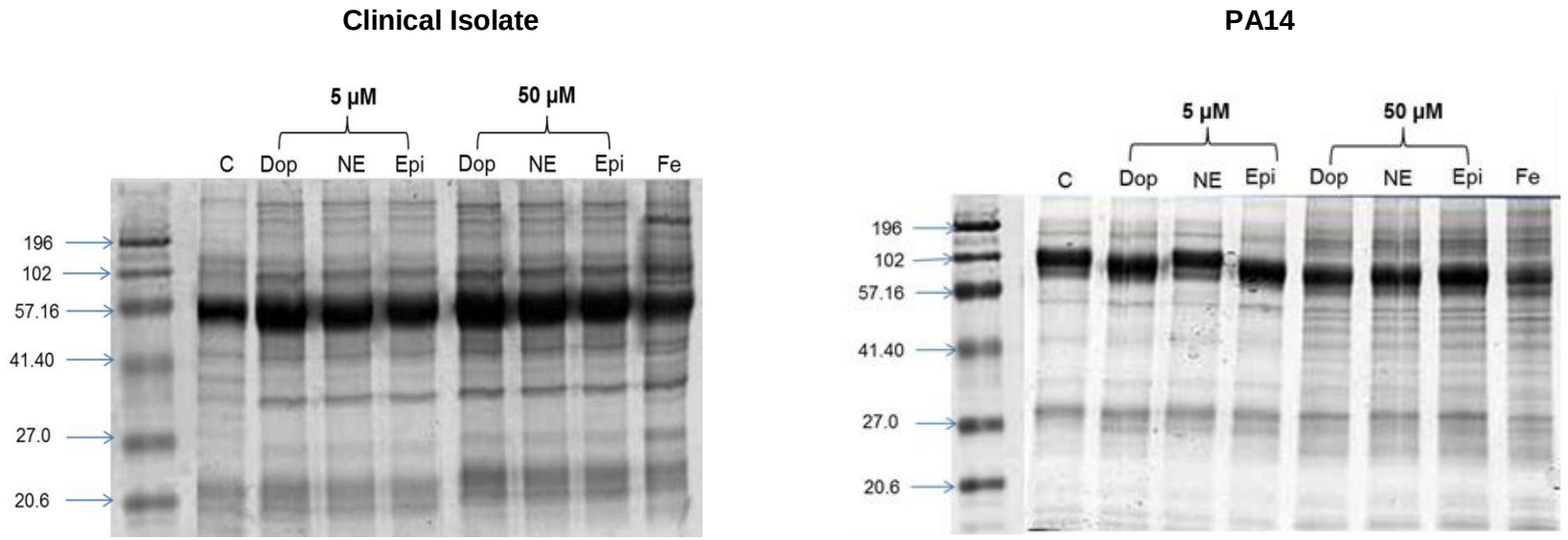


Figure 3.15 SDS-PAGE whole cell proteins profiles of inotrope and Fe-treated planktonic CI and PA14 strains:

P.aeruginosa grown in presence or absence of 5 µM, 50 µM of catecholamine inotropes Fe (50 µM) in serum-SAPI for 18 hrs in a CO_2 incubator at 37°C The proteins (25 µg of protein loaded per lane) were separated on 12% [vol/vol] acrylamide gels, and stained with coomassie stain. Representative gels of triplicate experiments. Keys: Dopamine (Dop), Norepinephrine (NE), Epinephrine (Epi).

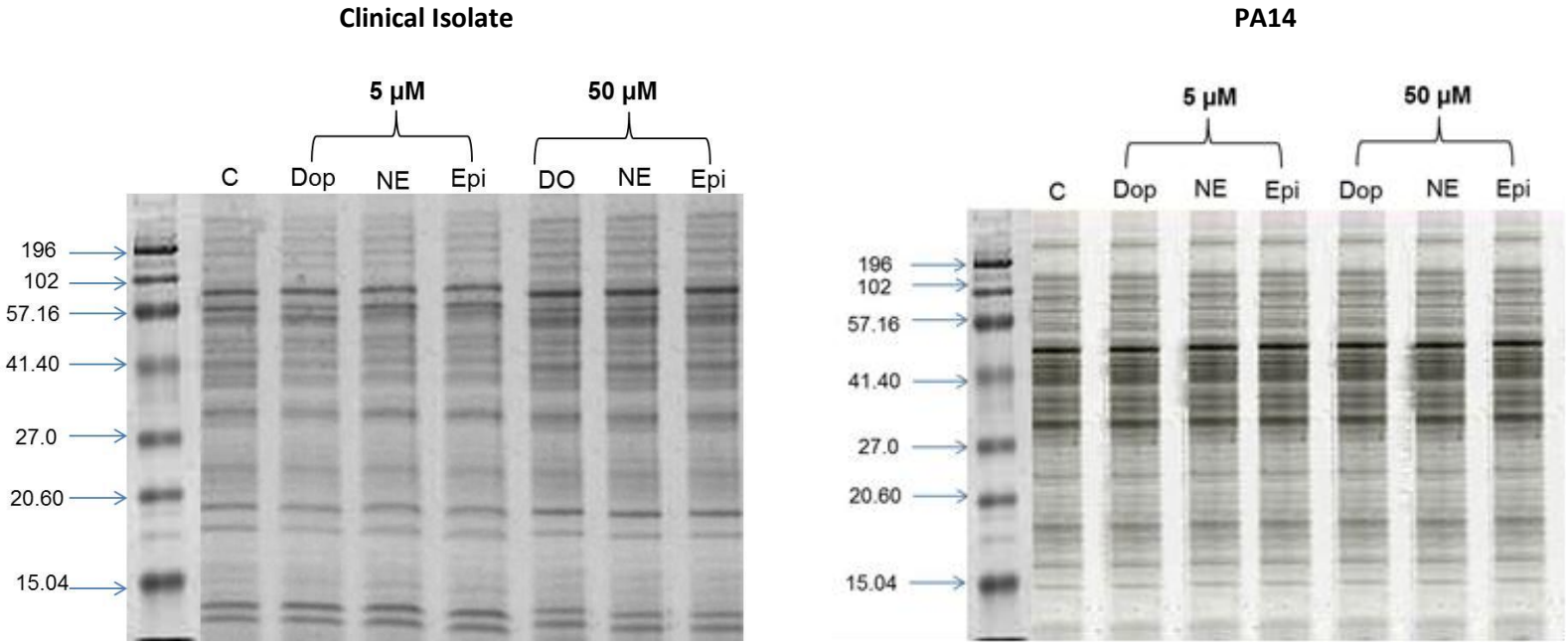


Figure 3.16 SDS-PAGE whole cell protein profiles of inotrope treated clinical isolate and PA14 biofilm bacteria (48h).

Bacteria were grown in serum-SAPI in the presences of the concentrations of the catecholamines shown and proteins were extracted as described in section 3.2.6. The proteins (25 µg of protein loaded per lane) were separated on 12% [vol/vol] acrylamide gels, and stained with coomassie stain. Representative gels are shown which are typical of three separate experiments. Keys: DO-Dopamine, NE- Norepinephrine, Epi- Epinephrine.

Table 3.2 Proteins identified in the presence of Norepinephrine (5µM & 50 µM) in *P.aeruginosa* Clinical Isolate (P-Plankton, B-Biofilm)

Protein Identified	Accession Number	KDa	Function	Control (P)	+NE (P) (5 & 50 µM)	Control (B)	+NE (B) (5 & 50µM)
Ferric uptake regulation protein (Fur)	B7V1H6	15	Iron uptake regulation	-	+	-	+
Adenosylhomocysteinase	B7V419	51	May play a key role in the regulation of the intracellular concentration of adenosylhomocysteine	-	+	+	+
4- hydroxyphenylpyruvate dioxygenase	B7UY69	40	Iron and metal ion binding	-	+	-	+
Nitrous-oxide reductase	B7V129	71	Nitrous oxide reductase activity, Ca^{2+}, copper ion binding	+	-	+	-
ClpB protein	B7V085	95	Chaperone which is involved in response to heat	-	+	-	+
DNA-directed RNA polymerase subunit beta	A6UZI1	151	DNA binding	-	+	-	+
Flagellin type B	B7UX97	49	Flagellar motility	-	+	-	+
Aconitate hydratase	B7UVG1	99	Iron and Sulfur binding	-	+	-	+
Nitrite reductase	Q02TP7	63	Electron carrier activity, heme binding	+	-	+	-
Putative binding protein component of ABC transpor t	Q02GU5	60	Transport activity	-	+	-	+
Glutamine synthetase	A6VDN7	52	Nitrogen metabolism	-	+	-	+
Aromatic-amino-acid aminotransferase	P43336	43	Aromatic amino acid biosynthesis	-	+	-	+
Putative universal stress protein	B7UZC9	31	Stress response	-	+	-	+
Outer membrane protein OprF	A6V748	38	Porin activity	-	+	-	+
Alginate regulatory protein	Q02EB0	35	Alginate production	-	+	-	+
Chitinase	Q02M97	53	Carbohydrate binding, Quorum sensing regulation	-	+	-	+

3.3.8 *P. aeruginosa* interaction with non-catecholamine inotropes

An investigation was also carried out using the non-catecholamine inotropic agents phenylephrine (neosynephrine) and vasopressin to see whether these drugs had any stimulatory effects on *P. aeruginosa* strains.

As shown in Figures 3.17 and 3.18 neither drug had any effect on growth or biofilm formation at any of the concentrations tested. Other aspects of *P. aeruginosa* virulence such as pyoverdine production, or swimming and twitching motility were also unaffected by the levels of phenylephrine and vasopressin (data not shown).

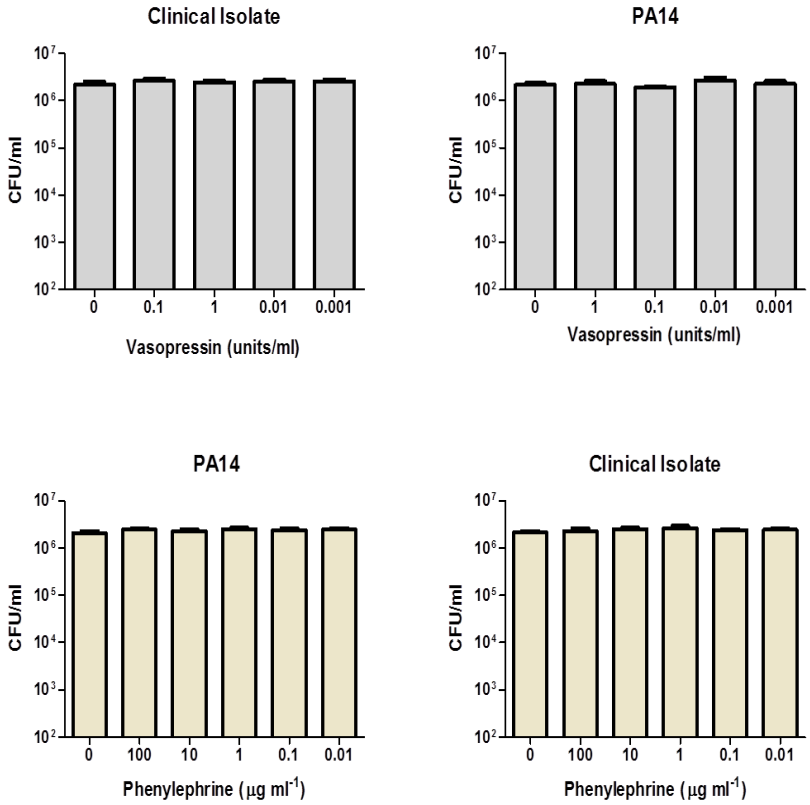


Figure 3.17 Effects of the non-catecholamine inotropes vasopressin and phenylephrine on *P. aeruginosa* growth

The panels shows the effects of a range of vasopressin concentrations on growth of *P.aeruginosa* strains CI and PA14 after 18 hrs incubation (n=4).

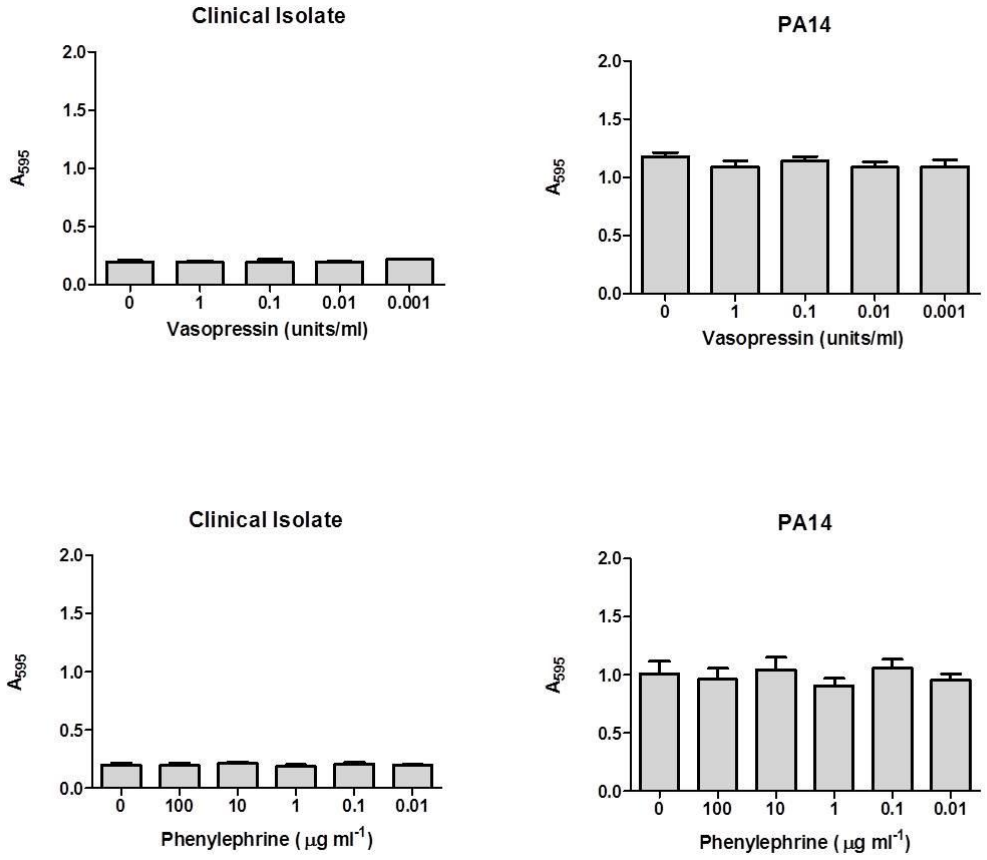


Figure 3.18 Effects of the non-catecholamine inotropes vasopressin and phenylephrine on *P. aeruginosa* biofilm formation.

(A) The panels show the effects of a range of vasopressin and phenylephrine concentrations on biofilm formation *P.aeruginosa* strains Clinical Isolate and PA14 (n=4). The results were not statistically significant (P>0.05).

3.4. Discussion

Ventilator associated pneumonia (VAP) caused by hospital-acquired bacteria and fungi is a major cause of morbidity and death to intensive care patients, despite intensive research to reduce its incidence (Morehead and Pinto, 2000, Gaurev and Gomez, 2003). *P. aeruginosa* is responsible for a significant proportion of VAP infections.

Catecholamine-based inotropic drugs have been shown to induce growth of both pathogenic and commensal bacteria (Neal et al., 2001, Freestone *et al*., 2002) and stimulate biofilm formation in *S.epidermidis* (Lyte *et al*., 2003). Approximately 50% of patients in intensive care units (ICU) receive catecholamine inotropic support during their hospital stay (Smythe *et al*., 1993). The respiratory tissues, via the endotracheal tube, may also be used as a direct site for systemic administration of inotropes (Raymondos *et al*., 2000) and procedures such as ET tube suctioning of patients have shown to contribute to significant systemic increase in norepinephrine and epinephrine (Schmidt and Kraft, 1995). Catecholamines such as epinephrine may also be directly administered via the ET (Stannard and O'Callaghan, 2002). Dopamine and norepinephrine are also naturally present within respiratory mucus in mammalian animals (Lucero *et al.*, 1998). This suggests that the bacteria inhabiting the ET and upper airways are likely to come into contact with both endogenous and exogenous catecholamines. *P. aeruginosa* biofilms are important issues in the pathogenesis of bacterial associated VAP due to the relatively high resistance of biofilms bacteria to antibiotics (Adair *et al*., 1993, 1999) and endotracheal tube is a factor thought to influence ventilated patients in the development of pneumonia. Previous studies have mainly focused on cultivation, identification and antibiotic resistance of endotracheal tube biofilm bacteria (Inglis *et al*., 1989, 1995, Adair *et al*., 1999, Feldman *et al*., 1999).

In this chapter an investigation on the effects of catecholamine inotropes, widely used in the treatment of acutely ill ICU patients on *P.aeruginaosa* growth and biofilm formation was carried out. Also, a *P.aeruginosa* clinical isolate from a pneumonia patient was used to understand effect of inotropes on endotracheal tube biofilm formation.

All pathogenic bacteria require iron for growth *in vivo*, and for this reason iron restriction in host body fluids by iron binding proteins transferrin and lactoferrin is a primary host defence (Freestone *et al*., 2008). Catecholamine inotropes have shown to stimulate growth of a number of bacteria by scavenging iron from serum transferrin (Freestone *et al*., 2000, 2002, 2003 & 2007). Studies have shown that NE and other catecholamines stimulate *P. aeruginosa* growth at 50-100 µM concentration (Lyte and Ernst, 1992, Freestone *et al*., 1999, Belay and Sonnenfeld, 2002, O'Donnell *et al*., 2006). However, this study reveals that catecholamine inotropes could even significantly stimulate *P. aeruginosa* growth, at lower levels (5 µM) ($P<0.05$). (Figure 3.1). From this study it also became clear that the mechanism of growth stimulation by the catecholamines involved the catecholamine enabling *P. aeruginosa* to acquire and internalise the iron bound within transferrin, and resulted in internalisation of both iron and inotrope (norepinephrine) (Figures 3.2 – 3.5).

In response to iron limitation when within its host *P. aeruginosa* produces two structurally unrelated high and low affinity siderophores, pyoverdine and pyochelin, which mediate iron transport via membrane receptors (Ankenbauer and Quan, 1993). Pyoverdine is by far the key siderophore of *P.aeruginosa* and has been shown to stimulate growth in human serum/plasma and iron-transferrin-containing medium (Ankenbauer *et al*., 1985, Ratledge and Dover, 2000). Pyoverdine production in both *P.aeruginosa* strains were found to be significantly up regulated by the inotropes

($P<0.05$) (Figure 3.6). Interestingly, even though providing iron for growth, the inotropes did not repress the production of pyoverdine. This suggests that while the levels of iron provided by inotropes is enough to enable growth of *P. aeruginosa*, Fe levels in the bacteria were not high enough to turn off the Fe regulated pyoverdine synthesis. The data above confirm the proteomics profiles of inotrope and iron treated bacteria suggesting that although bacteria grown in presence of inotropes can acquire enough iron from transferrin to grow, at low concentration the amounts of iron they are internalising is not enough to affect the expression of certain iron regulated proteins. However, at higher concentration inotropes showed similarity in protein expression patterns (Figure 3.15). Interestingly, the 15 KDa Ferric uptake regulation protein (Fur) was identified only with NE treatment in both planktonic and biofilm protein samples (Table 3.2). The Fur protein governs iron acquisition and siderophore mediated, iron transport systems in many bacteria (Hantke, 1987) including *P.aeruginosa* (Lewin *et al*., 2002; Hassett *et al*., 1996). Banin and co-workers (2005) have suggested that a critical level intracellular iron serves as the signal for biofilm development which is mediated by Fur in *P. aeruginosa* (Banin *et al*., 2005). The current finding confirms the role of inotropes in the facilitation of iron acquisition from transferrin in both planktonic and biofilm stages (Figures 3.14 and 3.15). By increasing the intracellular iron levels inotropes might be expected to inhibit expression of Fe-regulated genes in *P. aeruginosa* (Ratledge and Dover, 2000).

The cell surface and the substratum characteristics of bacteria are important for the initial attachment stage of biofilm formation. The presence of flagella, pili, EPS all influence the rate of initial attachment (Donlan, 2001). Flagella and type IV pili, Cup fimbria and pel gene and alginate are the most frequently cited *P. aeruginosa* determinants among those shown to be implicated at various stages of biofilm

formation (Sauer *et al*., 2005, Mace *et al*., 2008; O'Toole and Kolter, 1998 a, b). O'Toole and Kolter (1998) have proposed that flagellar mediated motility brings *P aeruginosa* closer to a surface, and to overcome the repulsive forces between the bacterium and the surface to which type IV pili eventually adhere in more stable interaction that aids in microcolony formation. All three catecholamine inotropes significantly induced flagella mediated swimming motility of both strains tested ($P<0.05$) (Figure 3.10). Interestingly, catecholamine inotropes significantly altered the twitching motility ($P<0.05$) of the Clinical Isolate but PA14 twitching was less and not affected by the drugs (Figure 3.11). This could be a strain difference or media-related effect. As shown in the fluorescence microscopic images (Figure 3.8) the inotrope-treated clinical isolate strain formed larger micro colonies whereas, PA14 had small aggregates densely spread on the surface. This finding suggests that inotropes influence twitching motility of VAP- causing *P.aeruginosa* thereby enhancing adherence, colonisation and biofilm formation onto surfaces such as endotracheal tubes. The microscopic analysis of the biofilm matrix (EPS) also suggests that inotropes may play a role in the EPS production (Figure 3.14). Although, the endotracheal tubes are made of PVC (polyvinyl chloride) most biofilm experiments were conducted on polystyrene in this study and it is assumed that there might not be any major difference in relation to the surface properties, as the reference strain *P. aeruginosa* PA14 has been shown to form similar degree of biofilm formation on both polystyrene and PVC (Friedman and Kolter, 2004a). Based on the above findings it is clear that the catecholamine inotropes (dopamine, norepinephrine and epinephrine) play an important role in biofilm formation and subsequent development of *P.aeruginosa* and may contribute to the endotracheal tube biofilm formation.

The non-catecholamine inotropes vasopressin and phenylephrine in contrast to the catecholamines, did not have any stimulatory effect on growth and virulence of the *P. aeruginosa* strains at the concentrations tested. This further confirms that the drug effects observed were seen only with the catecholamine structure inotropes (Figures 3.17, 3.18).

This study has revealed that those inotropes (dopamine, norepinephrine and epinephrine) that are most frequently administered to ventilated patients can all increase *P. aeruginosa* biofilm formation on endotracheal tubes (Figure 3.9). These results suggest that there could be a causal connection between inotrope medication and biofilm development in VAP. The clinical isolate (CI) from the pneumonia patient showed stable biofilm formation and a differential effect in 48 hours with the inotropes. It is evident from previous studies that nosocomial pneumonia occurs after 48 h of endotracheal intubation (Alp and Voss, 2006, Rewa and Muscedere, 2011), the time required for the inotropes to stimulate biofilm formation in the *P. aeruginosa* strains in this study. This suggests there may be a causal connection between endotracheal tube biofilm formation and inotrope medication.

To conclude, this chapter has shown that *P. aeruginosa* is using catecholamines as an environmental signal to initiate biofilm formation. Those inotropes that are mostly widely used in intensive care settings, the catecholamines, all significantly increased the growth and biofilm formation of the *P. aeruginosa* strains investigated ($P<0.05$). These results suggest that administration of inotropes to patients in intensive care, particularly if high doses are given, could indeed be a factor in the development of VAP by *P. aeruginosa*. Also, this study reveals that catecholamine inotropes affect the protein expression patterns and thus may be involved in the differential regulation of *P. aeruginosa* genes. However, further studies such as two-dimensional gel

electrophoresis proteomic analysis and microarray analysis have to be carried out to confirm this finding.

Chapter Four

Analysis of the effects of catecholamines on biofilm formation by Enteric bacteria

4.1 Introduction

Recent research is increasingly showing that bacteria and their hosts communicate with each other through hormone-like compounds. This communication is referred to as interkingdom cell-to-cell signalling (Freestone *et al.,* 2008, Hughes and Sperandio, 2008, Lyte and Freestone, 2010, Sharaff and Freestone, 2011). Bacteria sense and respond to environmental signals via bacterial signalling mechanisms, such as two-component signal transduction systems, global transcriptional regulators and quorum sensing systems (Laub and Gaulian, 2007). Many species of bacteria regulate gene expression in response to quorum sensing which has been discussed (section 1.2.1). Many pathogens sense and respond to the host adrenergic signalling molecules epinephrine and norepinephrine to recognize the host environment and promote the expression of virulence factors (Lyte, 2004, Freestone *et al*., 2008).

Quorum sensing has been shown to be a global regulatory mechanism in enterohemorragic *E. coli* (EHEC), involved in AE lesion formation, motility, metabolism, growth, and Shiga toxin expression (Sperandio *et al*., 2001). Preliminary evidence for a possible connection between quorum sensing and interkingdom signalling comes from the discovery of the epinephrine and norepinephrine/autoinducer-3 (Epi/NE/AI-3) QS system was revealed during an investigation of the regulation of virulence gene expression in EHEC O157: H7 (Sperandio *et al.,* 2003) the synthesis of the AI-3 molecule is thought to require LuxS. Sperandio et al. (2003) have shown that the enteric pathogen EHEC senses AI-3 produced by the microbial gastrointestinal flora to activate the expression of virulence genes, encoded by the chromosomal pathogenicity island locus of enterocyte

effacement (LEE) which leads to the formation of attaching and effacing (AE) lesions (explained in detail in Chapter One section 1.4.2). In addition, it was reported by this group that catecholamine hormone epinephrine was able to substitute for a mutant lacking AI-3 and activate the expression of virulence genes in EHEC.

Further work by Clarke et al. (2006) has shown that Epi/NE/AI-3 crosstalk is mediated by a membrane-embedded sensor histidine kinase named QseC. The QseC sensor kinase has also been proposed to sense specifically bacterial AI-3 as well as the host hormones epinephrine and norepinephrine, by binding directly to these signals. Upon sensing AI-3/Epi/NE in the gastrointestinal tract, the sensor kinase QseC is activated and undergoes autophosphorylation; subsequently, QseC transduces the signal to its cognate response regulator QseB, which activates the transcription of the master regulator of the flagella regulon, flhDC and genes involved in AE lesion. Expression of LEE, shiga toxin, flagellar and motility genes are thought to be regulated by the signals AI-3/Epi/NE through QseC (Clarke *et al*., 2005, Clarke *et al*., 2006, Hughes *et al*., 2009)

In another study on the basis of biochemical evidence it was shown that QseBC constitutes a functional two-component signal transduction system (Clarke and Sperandio, 2005). It was also proposed that the QseBC system shares homology with *Salmonella enterica* serovar Typhimurium PmrAB, and that it was involved in regulation of flagella and motility in EHEC (Sperandio *et al*. 2002b). In addition, to prove that QseC is indeed a bacterial adrenergic receptor, it was shown that the binding of the above signals by QseC was blocked by the administration of the α-adrenergic antagonist phentolamine (Clarke *et al*., 2006).

Recently, a second novel two-component system in the AI3/epinephrine/norepinephrine signalling cascade has been described by Reading et al. (2007) and named QseEF. QseE is the sensor kinase, and QseF the response regulator. QseF activates transcription of the recently described gene encoding EspFu (Reading *et al*. 2007), an effector protein of EHEC, which is encoded outside the LEE region. Transcription of qseEF is activated by epinephrine, phosphate and sulphate sources through QseC. Reading et al. (2007) then showed that the QseEF two-component system, involved in espFu transcriptional activation, is required for AE lesion formation for pedestal formation and actin polymerisation.

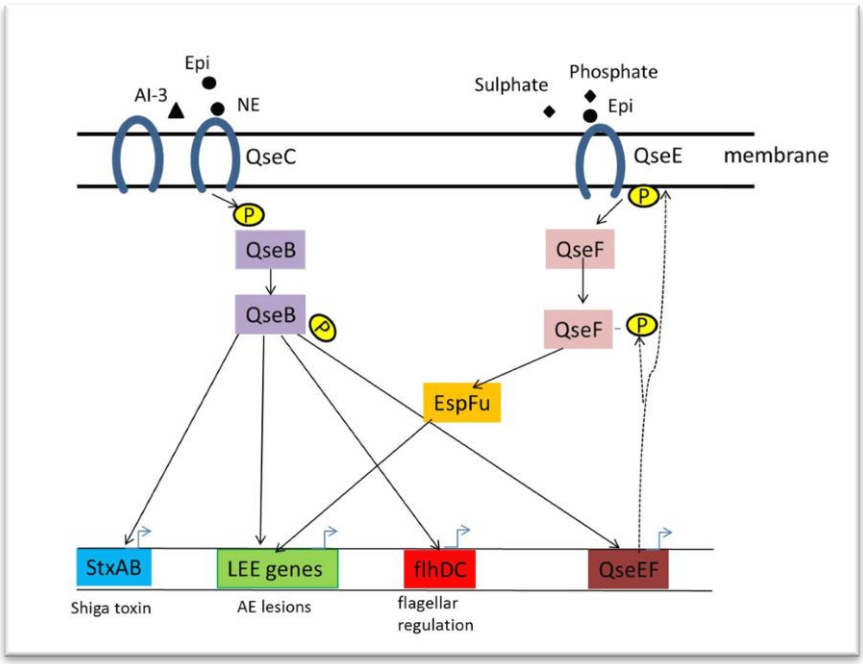


Figure 4.1 Model of the QseC and QseE signaling cascades in EHEC derived from Hughes and Sperandio, (2008) and Hughes et al. (2009). Autoinducer (AI)-3, epinephrine and norepinephrine (NE) bind the bacterial membrane receptor QseC, which results in its autophosphorylation. QseC then phosphorylates its response regulator QseB and initiates a complex signalling cascade that activates the expression of a second two-component system (QseEF), the locus of enterocyte effacement (LEE) genes (which encode various proteins necessary for attaching and effacing (AE) lesion formation the motility genes (flhDC) and Shiga toxin (stxAB).

QseC was demonstrated to play an important role in pathogenesis *in vivo*. By using a rabbit infection model, *qseC* mutants were shown to be attenuated for virulence (Sperandio *et al*., 2003, Waldor and Sperandio, 2006, Clarke *et al*., 2006). *In vivo* studies provide evidence that QseC histidine sensor kinase plays a role in the virulence of *Salmonella* Typhimurium (Bearson and Bearson, 2008) and *Francisella tularensis* (Weiss *et al*., 2007), since *qseC* mutants of these strains were attenuated in animal models.

The QseBC signalling system is not restricted to EHEC and other *E. coli*. In silico analyses have found homologs of QseC in about 25 bacterial species which includes *Salmonella* sp., *Shigella flexneri, F. tularensis, Haemophilus influenzae, Erwinia carotovora, Pasteurella multocida, Ralstonia eutropha* and *Chromobacterium violaceum*. The QseEF system is also found in other bacterial species such as *Salmonella spp*., *Shigella spp*. However, it is restricted to enteric bacteria unlike the QseBC system (Rasko *et al*., 2008). EHEC, *Salmonella*, *Shigella* and other food-borne pathogens have stimulated increased interest in the understanding of the pathogenic mechanisms of these deadly organisms due to their clinical and epidemiological significance. Rasko et al. (2010) has proposed that inhibition of interkingdom intercellular signalling constitutes an effective strategy for the development of a novel set of antimicrobial agents (Rasko *et al*., 2010).

Bearson and co-workers (2008) have studied the gene expression profile of *Salmonella enterica* serovar Typhimurium to norepinephrine. This study showed from microarray analysis that the flagellar genes fliA, fliY and fjB were upregulated in the presence of norepinephrine which was confirmed by motility assays. In addition, they constructed a *qseC* mutant and showed that the mutant had decreased motility compared to the wild

type and suggested the role of Qse sensor kinase in the modulation of *Salmonella* motility. Furthermore, they showed that the *qseC* mutant had decreased ability to colonize the gastrointestinal tract of swines. Based on the above investigations the authors have proposed that the AI-3/epi/NE inter-kingdom signalling may be involved during *Salmonella* colonization *in vivo*. (Bearson and Bearson, 2008)

Re-creation of the QseC and QseE mutants in *Salmonella* and *E. coli* has led to challenges as to whether the Qse receptors are truly the receptors for catecholamine responsiveness; there is also questioning if these genes are indeed involved in motility and attachment. The work in this chapter has therefore concentrated on the *in vitro* phenotypic characterisations of the *qseC, qseE, qseEC* mutants of *Salmonella enterica* serovar Typhimurium and Enterohaemorrhagic *E. coli* compared with their wildtype parent strains. Phenotypic analysis of the motility and biofilm formation abilities of these mutants, as well as their growth characteristics, protein expression to a variety of stress hormones are studied. These experiments were also carried out to investigate whether there is an effect of these genes in the formation of biofilms because for certain bacteria, e.g. *P. aeruginosa*, quorum sensing can play a significant role in regulation of biofilm formation. Findings of the *S.Typhimurium* Qse mutants been published, and suggest that bacterial adrenergic sensors may not be important in interkingdom signalling in *Salmonella* (Pullinger *et al*., 2010).

Aim

To investigate if catecholamines are signals which stimulate enteric pathogen biofilm formation. Another objective is to determimine if QseC and QseE mutation affect *E.*

coli and *Salmonella* biofilm formation, and to study whether these proteins are true bacterial adrenergic receptors for catecholamines.

4.2 Specific Methods

4.2.1 Time course growth kinetics

Overnight cultures grown in LB were diluted (1:100) in various liquid culture media: LB, LB that is iron-restricted through use of the ferric iron chelator dipyridyl (300 μM), a nutritionally challenging M9 with 0.4 % glucose as carbon source, and DMEM; in all cases continuous time courses of growth at 37^{o}C were carried out. Optical densities of cultures were measured at 600nm and recorded every 15 min for 24h using a Varioskan spectrophotometer (Transgalactic Ltd). Growth curves shown were derived from triplicate time courses using three independent cultures.

4.2.2 Host Pathogen Interaction

The human epithelial cell line Caco-2 was originally derived from a human colon adenocarcinoma and is widely employed in studies of pathogen-host cell interactions because of its ability to form well-differentiated cell monolayers. This cell line is very similar to small intestinal enterocytes with respect to their structure, brush border enzymes, and time courses of differentiation. In terms of host-pathogen interactions, the Caco-2 cell line is widely used as an *in vitro* model for intestinal epithelium and is particularly good model for studying EHEC attachment. The Caco-2 cell lines used in this study were cultured and kindly provided by Dr.Richard Haigh, Department of Genetics, the University of Leicester.

4.2.2.1 Caco-2 cell culture

Caco-2 cells were purchased from Health Protective Agency: (http://www.hpacultures.org.uk catalogue number 86010202) with a passage of 45 cells. Caco-2 cells were grown in DMEM (with GlutaMAX™, 4500 mg/L D-Glucose, Sodium Pyruvate; GibcoBRL, 31966-047) supplemented with 20 % foetal bovine serum (heat inactivated; GibcoBRL, 10108-165) in 75 cm^2 flasks at 37° C in a humidified 5% CO_2 incubator. Cells were grown to 70% confluency and gently washed twice with 10 ml PBS. The cells were split by adding 0.25 % trypsin-EDTA and incubated for 2 mins at 37 °C; the culture flask was then banged hard to dislodge the monolayer to single cells. Immediately after dislodging, the cells were re-suspended in 10 ml of pre-warmed DMEM media and made up to a volume of 100 ml and then aliquoted to individual wells in 24 well plates (1ml/ per well) and incubated as stated above (a confluent 24-well (1.77 cm2) has 4.8 x 10^5 cells). After 2-3 days (to allow full settling) the cells were continuously re-fed every 2-3 days until the monolayer was confluent and there are domes across the entire monolayer which was approximately 7-8 days.

4.2.2.2. Adherence Assay

Cultures of *S.*Typhimurium and EHEC and the respective *qse* mutants were grown until stationary phase in LB medium at 37°C. Bacteria were harvested by centrifugation (5,000 x g, 10 min), and washed three times in 1 ml of DMEM and alternate rounds of centrifugation (5,000 x g, 10 min). Finally, the bacterial pellet was resuspended in an equivalent volume of DMEM. The re-suspended pellets were diluted to an OD_{600} of 0.1 and used to infect the Caco-2 cells, *S.*Typhimurium was used at a multiplicity of infection (MOI) of 100:1, while an MOI of 50:1 was used for EHEC; the infection

assays were then incubated for 3 hours at 37 °C in a humidified 5% CO_2 incubator. After incubation any non-adherent bacteria were removed by washing the cultures with PBS three times; for intracellular bacterial cell counts the Caco-2 cells were lysed with 0.5 ml 1% Triton X-100 (Sigma Aldrich, Poole, UK) in PBS. The lysed cultures (containing both attached and internalised bacteria) were serially diluted with PBS, and the dilutions plated on LB Agar; the bacterial CFU/ml counts over the 3 hour incubation were determined both in the presence and absence of the Caco-2 cells.

4.2.2.3. Invasion Assay

To measure the number of bacteria that had invaded the Caco-2 cells, the culture supernatant from each well containing the unbound bacteria was removed and washed with PBS, and then replaced with 0.5ml DMEM supplemented with 40µg/ml gentamicin. The culture was then incubated at 37° C for 1hr in a 5% CO_2 for 60 min to kill all extracellular bacteria. After incubation, the cells were washed with PBS to remove the gentamicin and the Caco-2 cells lysed with 1% Triton X-100 in PBS, and the viable internalised bacteria enumerated by serial dilution and plating onto LB agar as described for the adhesion assay above.

The total number of organisms bound to the Caco-2 cells was determined by performing the invasion assay but excluding gentamicin treatment. Since the modified version of the assay yielded the number of organisms bound plus internalized, the difference between the total number bound and the total number internalized was taken to give the number of bound organisms:

No of adhered bacteria (CFU) = Total number of bacteria associated to cells (CFU) – No of internalised bacteria (CFU)

4.2.3 Preparation and fractionation of proteins

*S.*Typhimurium, EHEC wildtype and the *qse* mutants were grown overnight in DMEM media, harvested by centrifugation at 8000 x g for 10 minutes at 4 ^{o}C and washed twice and then resuspended in envelope buffer (EB 10 mM Tris-HCl, pH 7.5). The protein suspensions were frozen overnight at –80°C, thawed and sonicated at 6-8 microns in 8 cycles of 15 second sonication followed by 45 second cooling. The lysed protein samples were centrifuged at 10,000 rpm for 10 minutes to remove cell debris and unlysed bacteria. The supernatant contained the whole cell proteins. The whole cell proteins extracts were centrifuged at 50,000 rpm for 10 minutes in a Beckman TL-100 ultracentrifuge to separate membrane proteins from cytoplasmic proteins. The supernatant of the ultracentrifuged extracts contained the bacterial cytoplasmic protein fraction, while the pellets contained the total membrane protein fraction. The cytoplasmic and membrane protein fractions were processed further for SDS PAGE as described in Materials and Methods (section 2.2.4)

4.3 Results

4.3.1 Analysis of whether the QSe mutation affect growth kinetics of *Salmonella* Typhimurium in different media

Overnight inocula of *S.*Typhimurium ST4/74 (Nal^R) and mutants Δ*qseC*, Δ*qseE* and Δ*qseCE* were prepared as described in Materials and Methods, and their growth kinetics examined in four different culture media (LB, iron restricted LB, DMEM and M9 minimal medium supplemented with 0.4% glucose) and compared with that of their wild-type parent (Figure 4.2). Similar growth profiles were observed between wild type and and Δ*qseC/E* mutants in LB but with addition of the iron chelator (dipyridyl) the growth was reduced and the wild type reached the stationary phase before the mutants. In DMEM medium the wild type *S.*Typhimurium parent also reached stationary phase before the mutants. In M9 minimal medium all the strains showed very similar growth profiles.

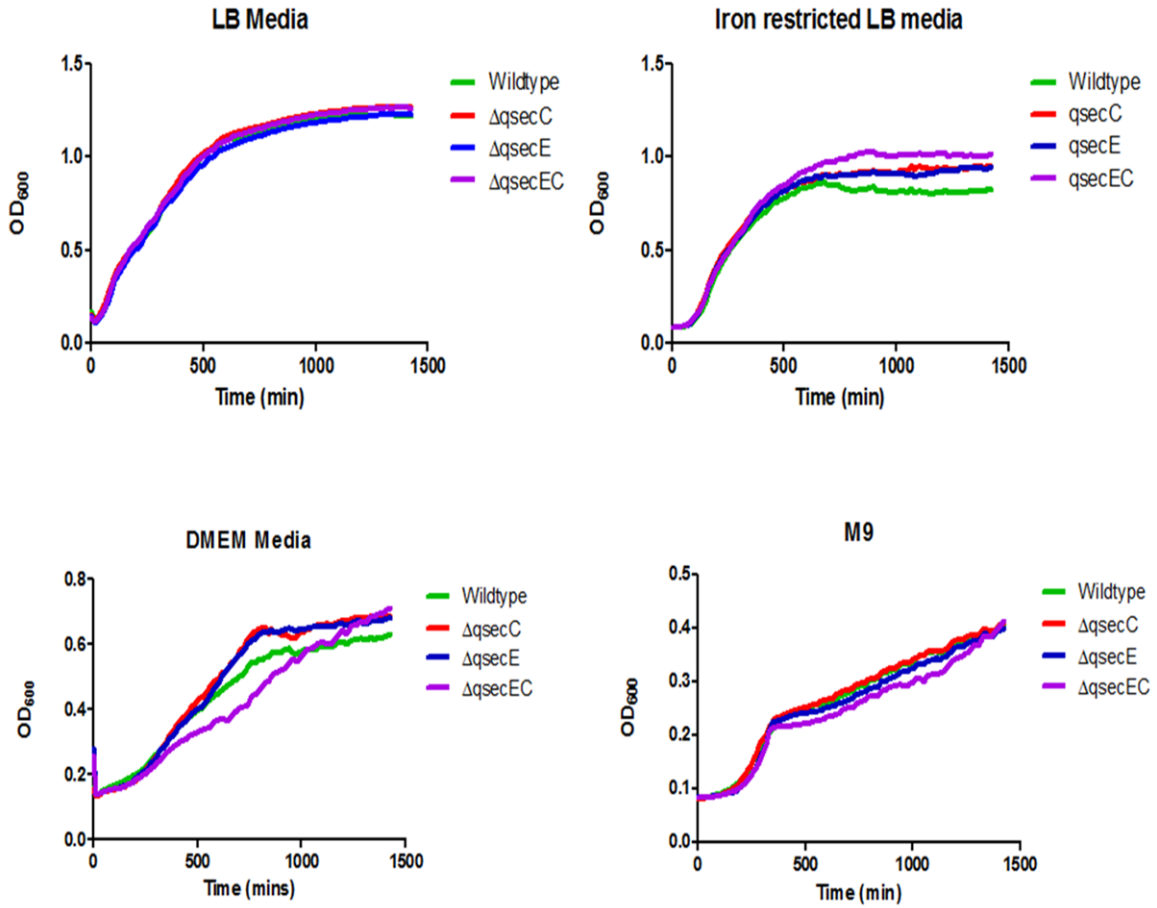


Figure 4.2 Growth kinetics of ST4/74 NalR and Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants in different media. Overnight cultures were diluted 1:1000 in LB, iron restricted LB, DMEM, and M9 minimal media. Growth kinetics was measured over 24 h as described in section 4.2.1

4.3.2 Catecholamine growth responsiveness of *S*.Typhimurium wild type and Qse mutants

The Qse proteins are proposed to be the *E. coli*/*Salmonella* receptors for the adrenergic stress hormones norepinephrine and epinephrine (Sperandio *et al*., 2003, Reading *et al*., 2009, Bearson and Bearson, 2008). Therefore, analyses of whether mutation of the QSe genes affected the ability to respond in the growth context to catecholamines were carried out. Response to the catecholamine stress hormones for all the strains as shown in Figure 4.3 was dependent on the initial cell density of the inoculating culture, and the difference in growth levels with and without the catecholamine was significant ($P<0.05$) at low cell densities but not significant ($P>0.05$) at the higher bacterial cell densities. In terms of recognising the catecholamines, overall, the Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants all exhibited a similar ability to respond to norepinephrine, dopamine and epinephrine as their wild-type parent. Epinephrine, which is not produced in the gut, was the least effective catecholamine at promoting growth of all the strains tested. These *in vitro* growth results clearly indicate that the putative adrenergic QseE and QseC sensor kinases play no role in *Salmonella* initial responses to the catecholamines.

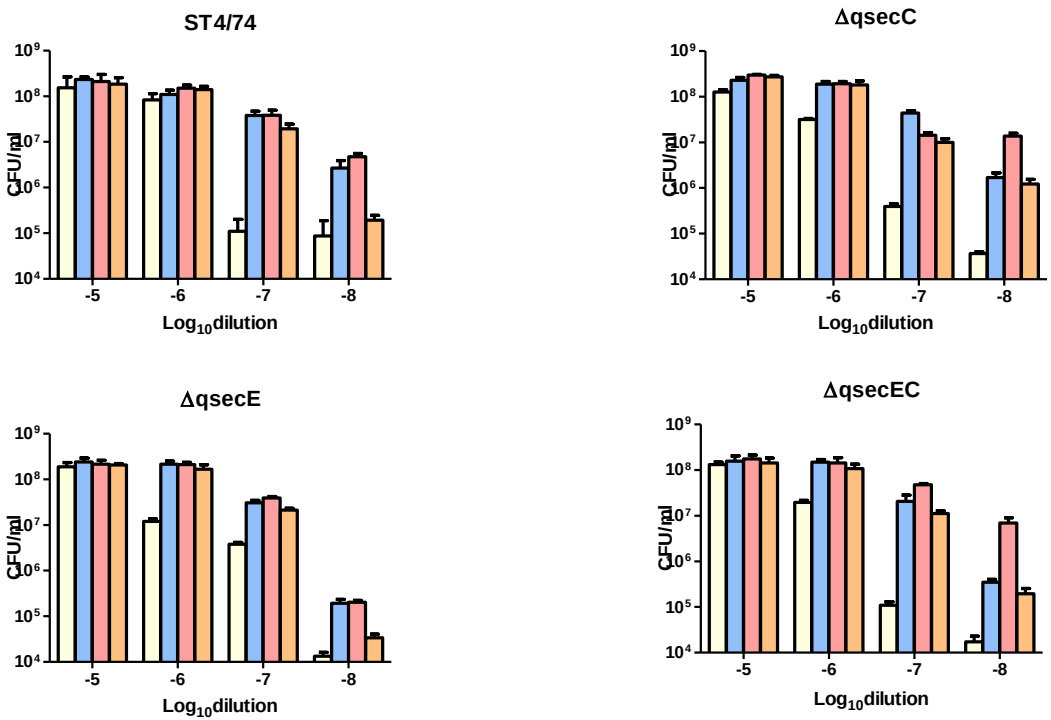


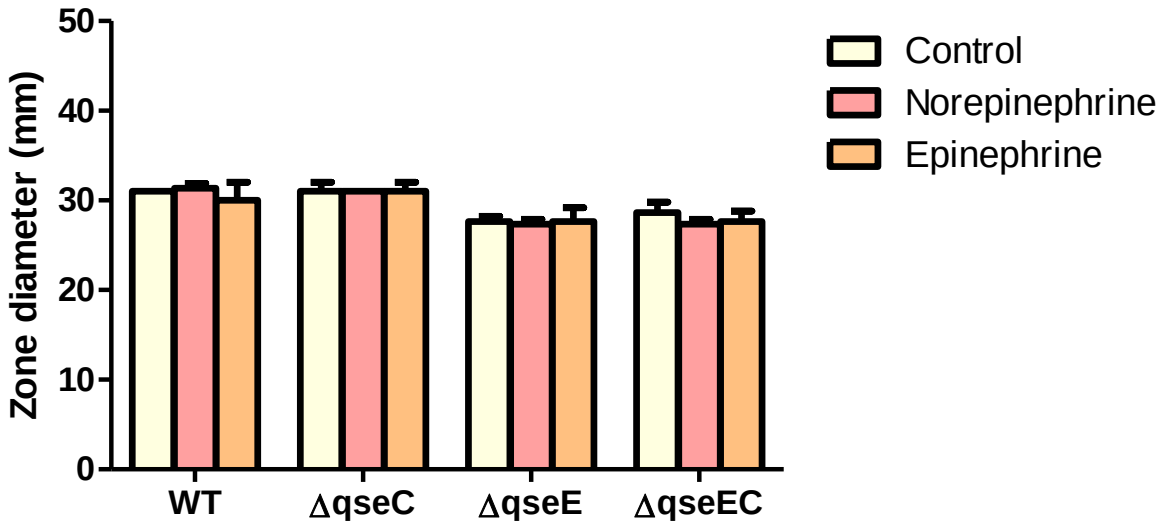
Figure 4.3 Effect of catecholamine supplementation on the growth of wild type ST4/74 Nal^R and Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants. Overnight cultures of the indicated strains (wildtype 6.73 x10^8 cfu/ml, Δ*qseC*, 6.93 x10^8 cfu/ml, Δ*qseE*, 4.60 x10^8 cfu/ml, Δ*qseCE* cfu/ml, 6.83 x10^8 cfu/ml) were diluted in 10-fold steps as shown on the x axes into serum-SAPI with or without the catecholamines shown (all at 100 µM), and grown for 18h statically at 37^{o}C. Bacteria were then enumerated by serial dilutions and plating. Pight yellow bars, no addition; pale blue, dopamine; dark pink, norepinephrine; pale orange, epinephrine. Values represent the means and standard deviations of triplicate platings from triplicate cultures (n=3). The differences between the strains were not statistically significant (P>0.05).

4.3.3 Motility Assays of *S*.Typhimurium wild type and *qse* mutants

The QseC and QseE proteins have been proposed to play a role in motility of *S.Typhimurium* (Bearson and Bearson 2008). The catecholamines (norepinephrine) have also been shown by these workers to apparently enhance motility in both wildtype and Δ*qse* mutants. Therefore, a comparative analysis was made as the motility of wildtype and *Δqse* mutants compared with each other, and in response to the adrenergic catecholamine norepinephrine and epinephrine.

The motility assays in Figure 4.4 show that the motility of the wildtype and *qseC* mutant were very similar; compared with wildtype, the *qseE* and *qseEC* mutants were somewhat less motile and all did not reach statistical significance ($P>0.05$). Inclusion of norepinephrine or epinephrine at either 5 or 50 μM did not affect the motility much of either wild-type *Salmonella* or the *qse* mutants.

A.



B.

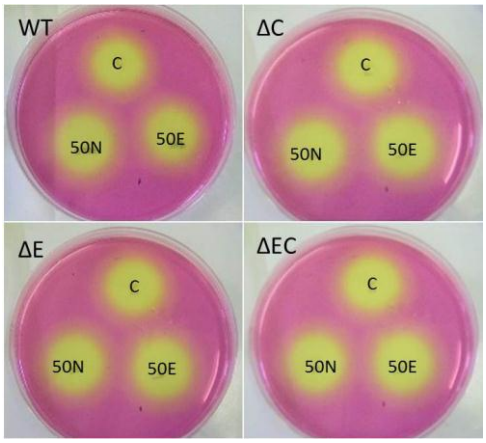


Figure 4.4 Swimming motility of ST4/74 wild-type and Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants in the presence and absence of 50 µM Norepinephrine or Epinephrine.

A, mean data showing motility of wildtype vs mutants at Norepinephrine and epinephrine concentration of 50 µM ($P>0.05$). **B**, photograph showing a typical motility assay. Key: WT, wildtype; ΔC, Δ*qseC* mutant; ΔE, Δ*qseE* mutant; ΔEC, Δ*qseEC* mutant. $P<0.05$

4.3.4 Biofilm formation of *S*.Typhimurium wild type and *qse* mutants

4.3.4.1 Biofilm formation

Biofilm formation of the wild type and the *qse* mutants was investigated as well as their biofilm formation to polystyrene in different media was tested (Figure 4.5, 4.6).

In DMEM medium only (the control) all the mutant strains showed similar levels of biofilm formation to wildtype. The addition of the catecholamines did not affect biofilm formation of the wildtype *Salmonella*. However, the *qseC* and the *qseE* mutant biofilm formation was slightly enhanced with the addition of norepinephrine and epinephrine respectively and did not reach statistical significance ($P>0.05$). The *qseEC* double mutant showed slightly more biofilm formation in the presence of all three catecholamines which was also not statistically significant ($P>0.05$) (Figure 4.5).

In serum SAPI medium biofilm formation in the control wells of the wildtype and *qseC* mutant was less compared to that of the *qseE* and *qseEC* mutants and was not statistically significant ($P<0.05$). The *qseC* and *qseE* mutant's biofilm formation was slightly increased by all three catechlamines, whereas the wildtype showed increase biofilm formation only in the presence of epinephrine and these differences were not statistically significant ($P>0.05$). The *qseEC* double mutant appeared to show reduced biofilm formation following the addition of all three catecholamines which was also not statistically significant ($P>0.05$) (Figure 4.6).

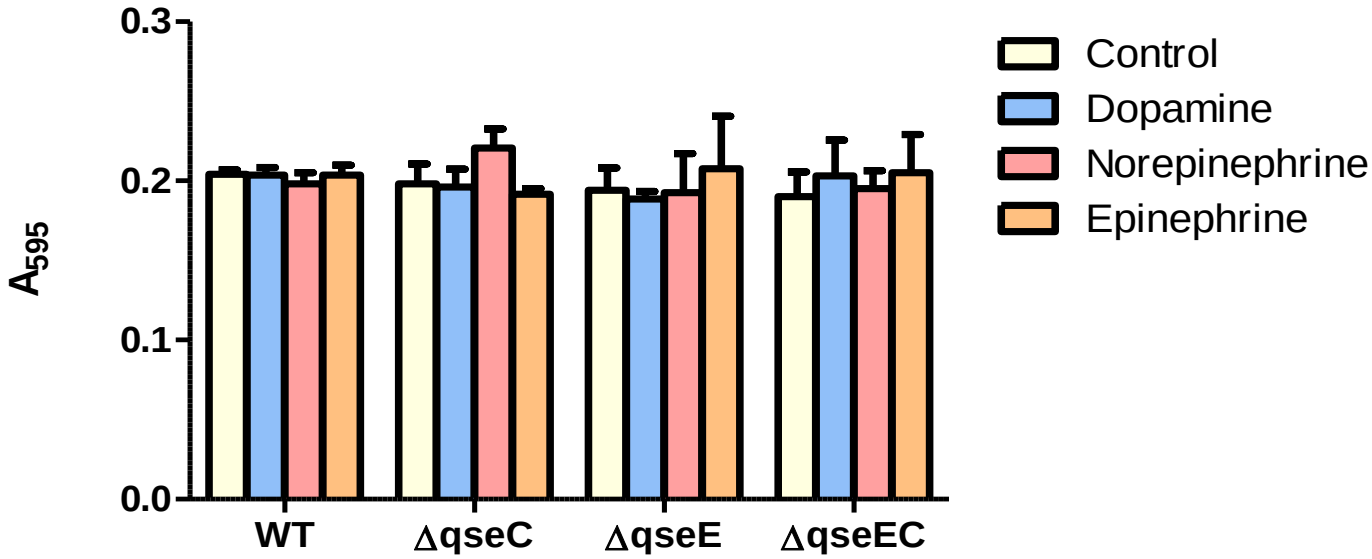


Figure 4.5 Biofilm formation of *S*.Typhimurium wild-type and *qse* mutants in DMEM media Overnight cultures were diluted 1:100 into the indicated media, and attachment measured at 48hrs after staining with crystal violet. Data represent bacterial attachment to a polystyrene surface with and without the addition of dopamine, norepinephrine and epinephrine (50µM); values shown are means and SD of 4 biological replicates from triplicate experiments. There were no significant differences with the catecholamine treatments ($P>0.05$).

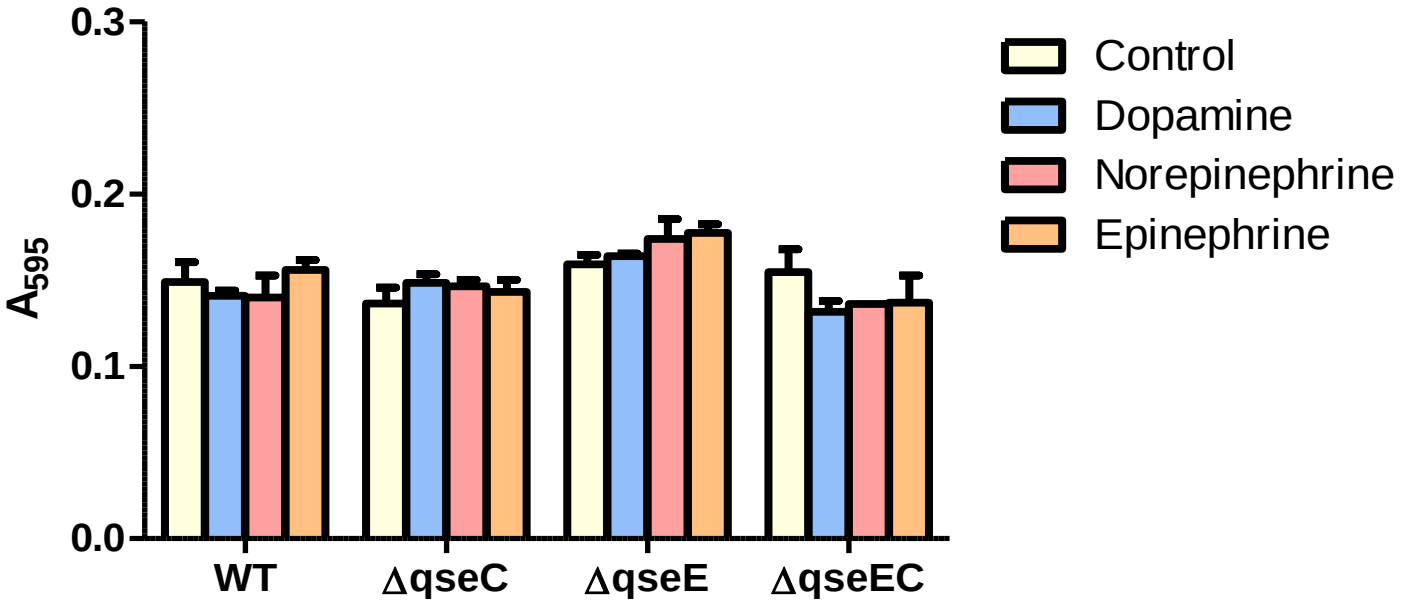


Figure 4.6 Biofilm formation of *S.Typhimurium* wild-type and *qse* mutants in serum-SAPI media Overnight cultures were diluted 1:100 into the indicated media, and attachment measured at 48hrs after staining with crystal violet. Data represent bacterial attachment to a polystyrene surface with and without the addition of dopamine, norepinephrine and epinephrine (50µM); values shown are means and SD of 4 biological replicates from triplicate experiments. There were no significant differences with the catecholamine treatments (P>0.05).

4.3.4.2 Microscopic examination of the biofilm formation of the *Salmonella* qse mutants

Direct microscopic analysis of biofilm formation on polystyrene microtiter plates was carried out by staining the wells with biofilm live dead fluorescent stain as described in Materials and Methods section 2.2.3.1 and 2.2.3.2. The stained well images were analysed and quantified by measuring the fluorescence emitted by the biofilm bacteria as integrated densities (Figures 4.7 and 4.8)

In DMEM medium (Figure 4.7A, B), the wildtype parent did not show much difference in biofilm formation following catecholamine treatment. However, the *qseC*, *qseE* and *qseEC* mutant strains biofilm formation was significantly more in the presence of epinephrine compared to the wildtype ($P<0.05$). *ΔqseC* and *ΔqseE* biofilm formation were significantly reduced with dopamine and norepinephrine ($P<0.05$). The double mutant Δ*qseEC* showed significant increase in biofilm formation with norepinephrine and decreased biofilm formation with dopamine ($P<0.05$).

Microscopic investigation of the mutant *qse* strain biofilm formation in serum-SAPI minimal medium (Figure 4.8 A and B) showed that the wildtype parent exhibited more biofilm compared to the mutants. Epinephrine stimulated biofilm formation of the wildtype, and *ΔqseE* mutant which did not reach statistical significance ($P>0.05$). NE significantly stimulated biofilm formation of both *ΔqseE* and *ΔqseEC* mutants ($P<0.05$). Dopamine stimulated biofilm formation in all three mutant strains though it also decreased biofilm formation of the wildtype. Dopamine effect on biofilm formation was not statistically significant ($P>0.05$).

Even though, there are differences in biofilm formation of the *S.* Typhimurium wildtype and mutants with catecholamine treatment in both mediums tested it cannot be confirmed that it is

due to QseC, QseEC mutation as the double mutant mostly showed better biofilm formation than the other strains.

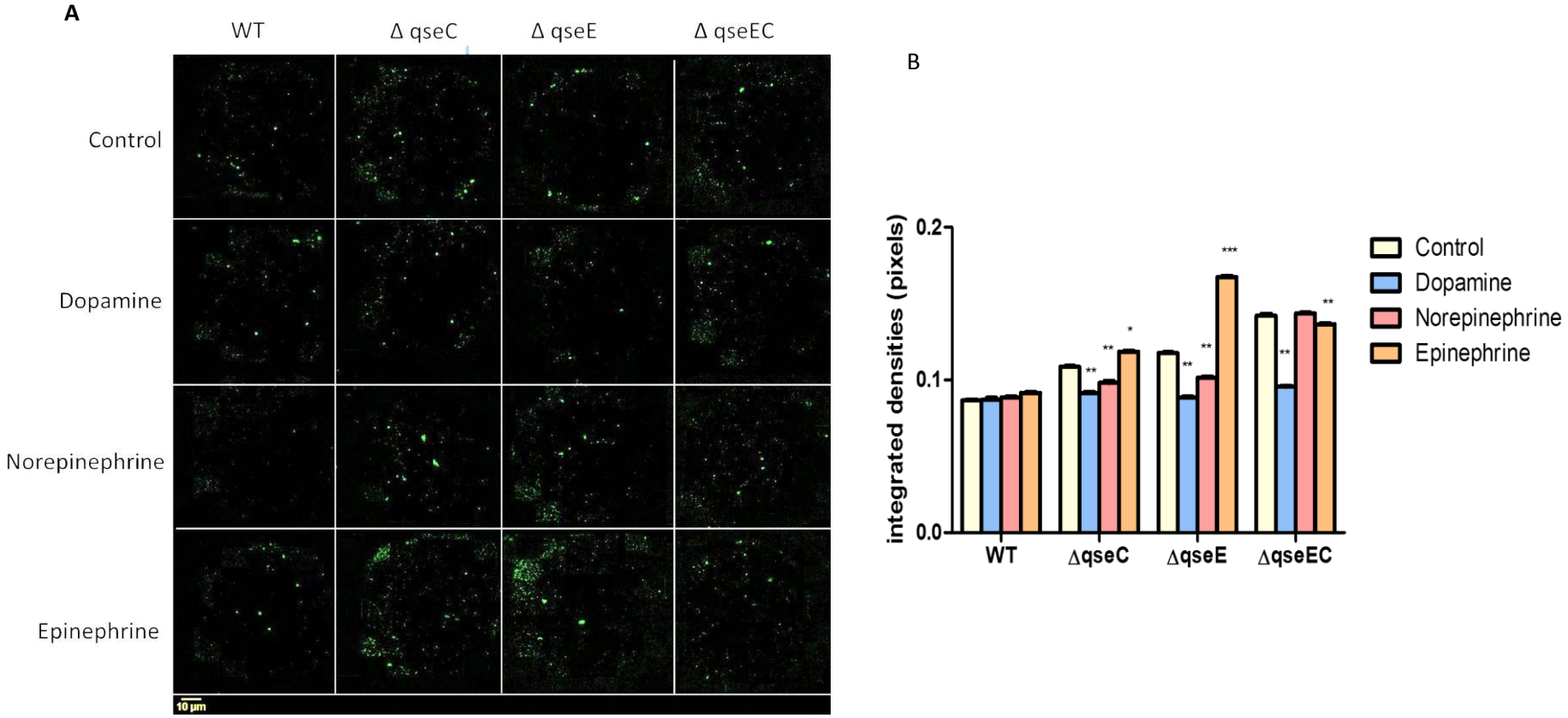


Figure 4.7 Microscopic analysis of biofilm formation of *S.Typhimurium* wild type and Δqse mutants in DMEM media

Bacteria were incubated for 48 hrs with addition of catecholamines (dopamine, norepinephrine, epinephrine). The 96 microtitre plate well bottoms were stained with biofilm live-dead stain were observed under NIKON Eclipse Ti inverted fluorescence microscope (10x). Biofilm formation is seen as small aggregates (green). B. Hisograms showing fluorescence intensities measured as integrated densities of biofilm bacteria for each well. Keys: The symbols * indicate statistical significance of * P<0.05, ** P<0.01; *** P<0.001

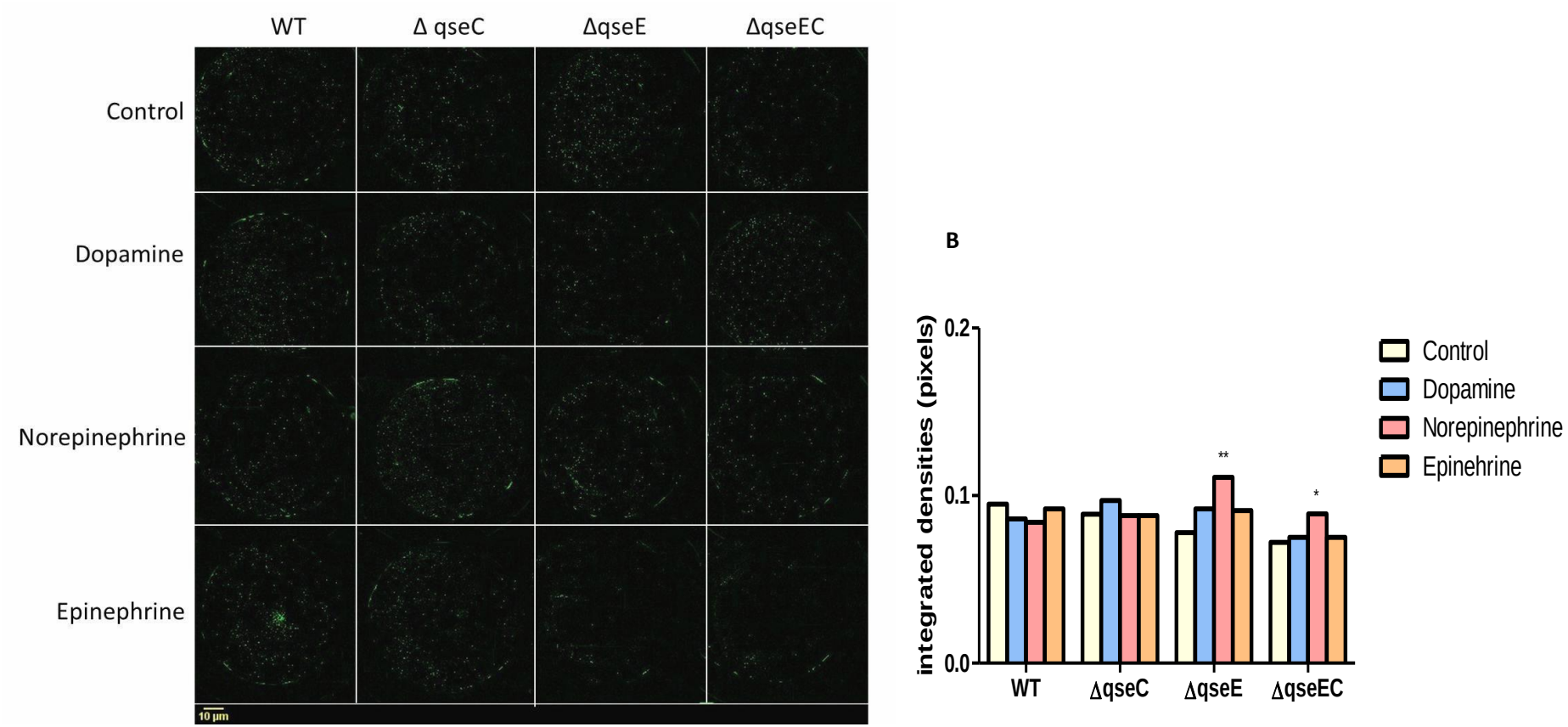


Figure 4.8 Microscopic analysis of biofilm formation by *S*.Typhimurium ST4/74 wild type and Δqse mutants in serum-SAPI media.

Bacteria were incubated for 48 hrs with addition of catecholamines (dopamine, norepinephrine, epinephrine). The 96 microtitre plate well bottoms were stained with biofilm live-dead stain were observed under NIKON Eclipse Ti inverted fluorescence microscope (10x). Biofilm formation is seen as small aggregates (green). B. Hisograms showing fluorescence intensities measured as integrated densities of biofilm bacteria for each well. Keys: The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$

4.3.5 Caco-cell attachment of *S*.Typhimurium wild type and *qse* mutants

Cultured intestinal cell lines are often used in bacterial attachment assays as indicators of the pathogenic potential of the bacteria in question. The ability of *Salmonella* to invade mammalian cells is important for its systemic spread and the development of enteritis in animals and humans (Watson *et al*., 1995). Previous studies have shown that the *qse* mutants were defective in their ability to attach and invade eukaryotic cells (Sperandio *et al*., 2003, Bearson and Bearson, 2008). It was therefore investigated whether the QseC, QseE mutation affected the ability of *Salmonella enterica* serovar Typhimurium to attach and invade Caco-2-cells.

Figure 4.9A shows that wildtype and the *Δqse* mutant strains all adhered to similar extents to the Caco-2 cells. However, *qseEC* mutant showed a significant reduction in adherence ($P<0.05$). The *qseC* mutant also showed a reduced level of adherence but was not statistically significant ($P>0.05$). Adherence was not affected by norepinephrine treatment (Figure 4.9).

The wildtype, and the *ΔqseC*, *ΔqseE* mutants were all similar in their ability to invade Caco-2 cells (Figure 4.9A). However, the double *ΔqseEC* mutant showed a significant 10 fold reduction in its ability to invade the Caco-2 cells ($P<0.01$). The invasive ability of the wild type and *qse* mutants were not affected by NE (Figuure 4.9).This suggests that the QseEC double gene mutation might play an important role in attachment and invasive ability to eukaryotic intestinal cell lines *in vitro*, though the mechanism by which this occurs is unclear.

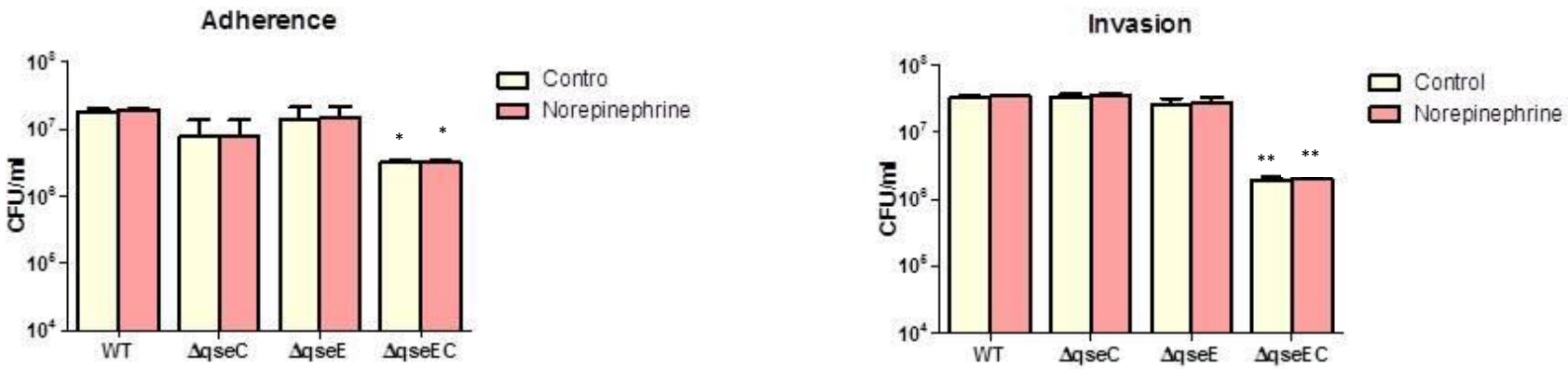


Figure 4.9 In vitro Caco-2 cell attachment of *S.*Typhimurium ST4/74 wild type and *Δqse* mutants in DMEM mediun

Initial inoculum was normalised to OD_{600} 0.1 and after 3 hrs incubation adherence and invasion was quantified as described in section 4.2.2. Histograms show a comparison of adherence and invasion ability of wildtype and *qse* mutants with and without 100 µM catecholamine treatment. The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.001$.

4.3.6 Proteomics investigation of *S.*Typhimurium wildtype and *qse* mutants

Protein expression of the *Salmonella* wild type and the *qse* mutants were studied by extracting the cytoplasmic and membrane proteins and running comparison SDS PAGE gels. The protein profiles of the wildtype and *qse* mutants showed essentially identical patterns (Figure 4.10 A,B), suggesting that deletion of the *qse* genes is not having a global effect on gene expression.

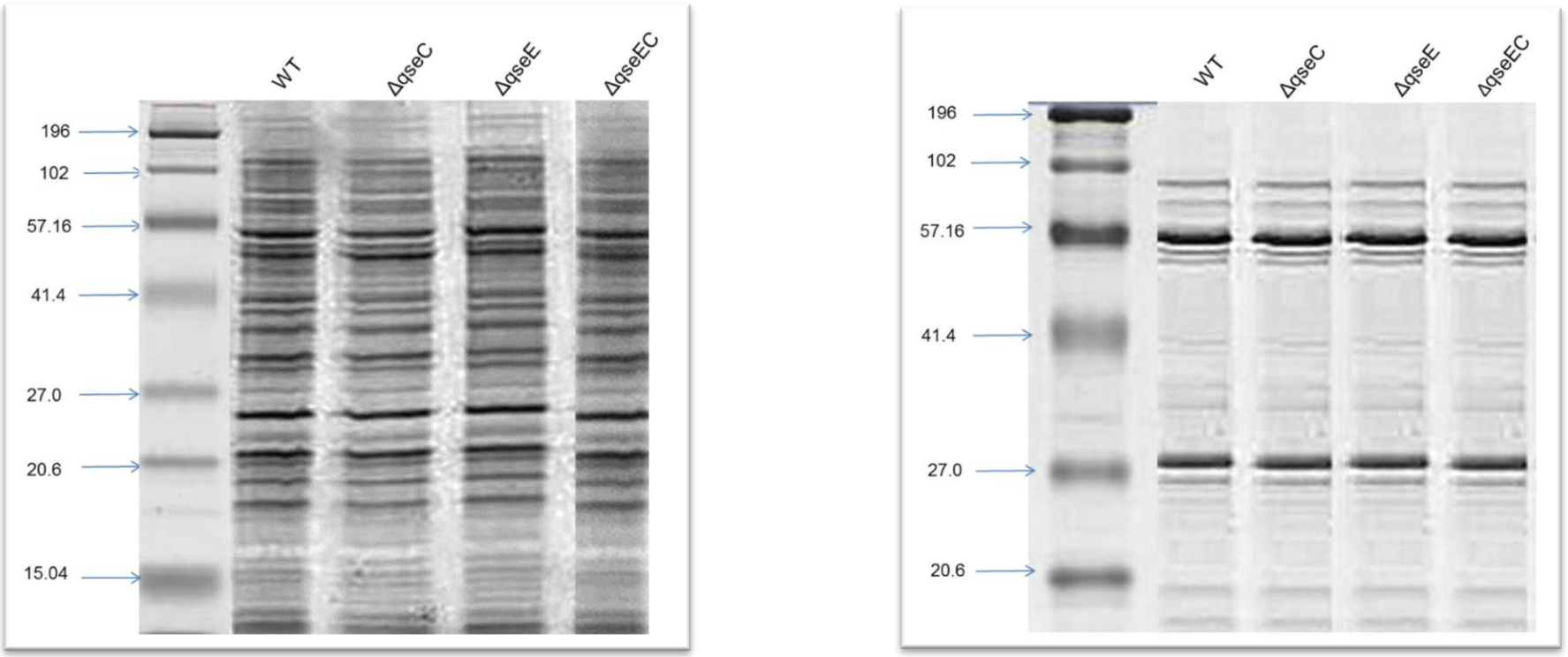


Figure 4.10 Proteomic investigations of *Salmonella* qse mutants. SDS-PAGE (12% [vol/vol] acrylamide) coomassie stain of cytoplasmic (A) and membrane proteins (B) of *S*. Typhimurium ST4/74 and Qse mutants grown in DMEM for 24 hrs; 30 µg of protein loaded per lane for cytoplasmic proteins and 30µg for membrane proteins; Molecular mass standards are shown on the left.

4.3.7 Analysis of whether the QSe mutation affects the growth kinetics of *E.coli* O157:H7

Cultures of EHEC strain (O157:H7) 85-170 (Nal^R) mutants Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants were prepared as described in Materials and Methods, and their growth kinetics examined in four different culture media (LB, iron restricted LB, DMEM or M9 with 0.4% glucose) and compared with that of their wild-type parent (Figure 4.11). The resultant time courses of the mutants all showed similar profiles to the parent strain (Figure 4.11). There were no differences between wild type and Δ*qseC/E* mutants when they were grown in LB only or with the addition of the iron chelator (dipyridyl). In DMEM medium growth defects were observed with the Δ*qseE* mutant, while in M9 medium the Δ*qseEC* double mutant had particularly slow growth relative to the other strains. This suggests that the Δ*qseEC* mutation may be causing metabolic defects within the *E. coli* cell.

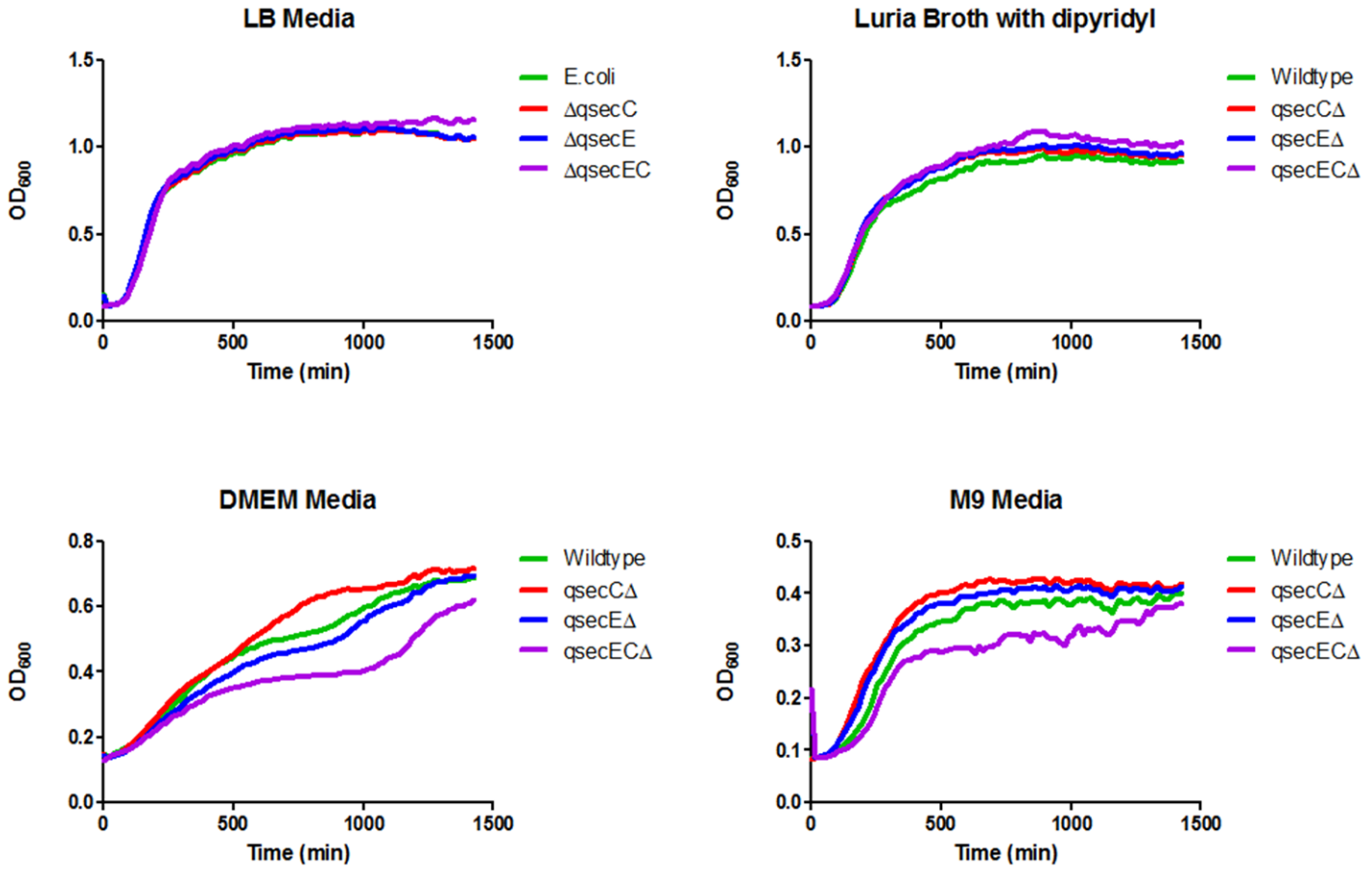


Figure 4.11 Growth kinetics of EHEC strain 85-170 and Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants in different media. Overnight cultures were diluted 1:1000 in LB, iron restricted LB, DMEM, and M9 minimal media. Growth kinetics was measured over 24 h as described in section 4.2.1.

4.3.8 Analysis of catecholamine growth responsiveness of *E.coli* O157:H7

Analysis of EHEC wildtype and the Δ*qse* mutants showed that the Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants all exhibited a similar ability to respond to the three catecholamines as their wild-type parent (Figure 4.12). These *in vitro* growth results confirmed that the Qse sensor kinases play no role in *E.coli* growth responsiveness to the catecholamines. Epinephrine was the least effective catecholamine at promoting growth of *E.coli* wild type and *qse,* mutants.

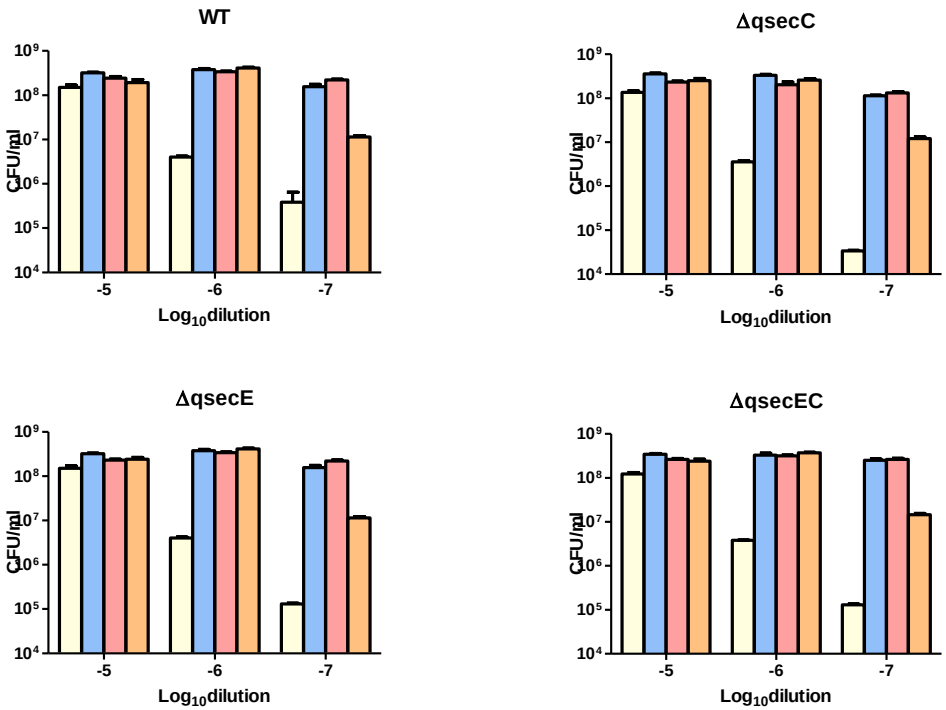


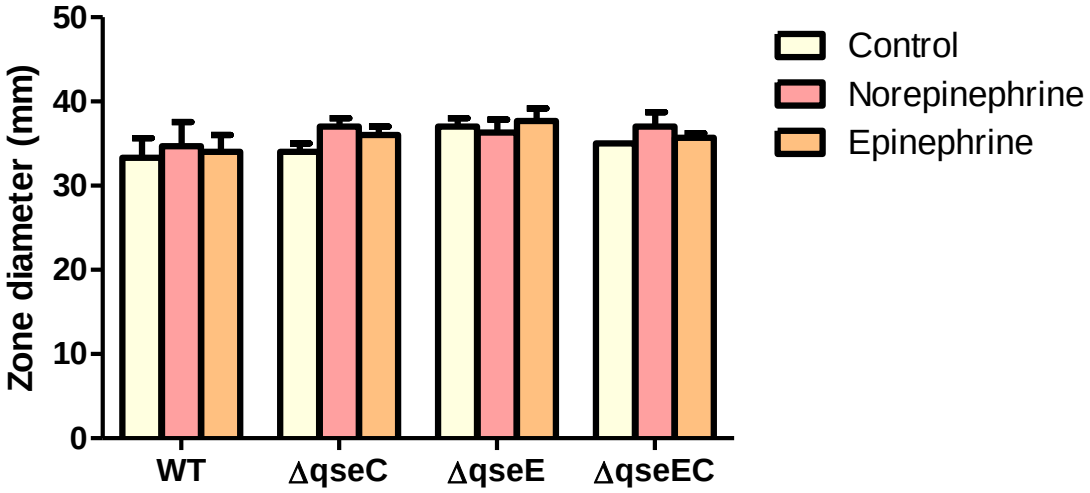
Figure 4.12 Effect of catecholamines on growth of EHEC (O157:H7) wildtype and Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants. Overnight cultures of the indicated strains (wildtype 4.5 x10^8 cfu/ml, Δ*qseC*, 4.6 x10^8cfu/ml, Δ*qseE*, 5.4 x10^8, Δ*qseCE*. 4.7 x10^8 CFU/ml) were diluted in 10-fold steps as shown on the x axes into serum-SAPI with or without the catecholamines shown (all at 100 µM), and grown for 18h statically at 37°C. Bacteria were then enumerated by serial dilutions and plating. Pale yellow bars, no addition; pale blue, dopamine; dark pink, norepinephrine; pale orange, epinephrine. Values represent the means and standard deviations of triplicate platings from triplicate cultures (n=3). The differences between the strains were not statistically significant (P>0.05).

4.3.9 Motility assays of *E.coli* O157:H7

Motility assays were performed to understand the role of QseC and QseE genes on motility in the presence and absence of norepinephrine and epinephrine. Both wild type and mutants formed similar motility halos with and without addition of the catecholamines (Figure 4.13). The difference between the wild type and the mutants in terms of motility was not significant ($P>0.05$). Importantly, mutation in Qse genes did not affect motility as has been proposed (Sperandio *et al*., 2003; Clark *et al*., 2006)

Addition of norepinephrine and epinephrine at 5 μM or 50 μM slightly increased the motility of wild-type *E.coli* and the *qse* mutants and was not statistically significant ($P>0.05$). The *ΔqseE* mutant showed increased motility with epinephrine addition and reduction in motility with norepinephrine but again this did not reach statistical significance ($P>0.05$).

A



B.

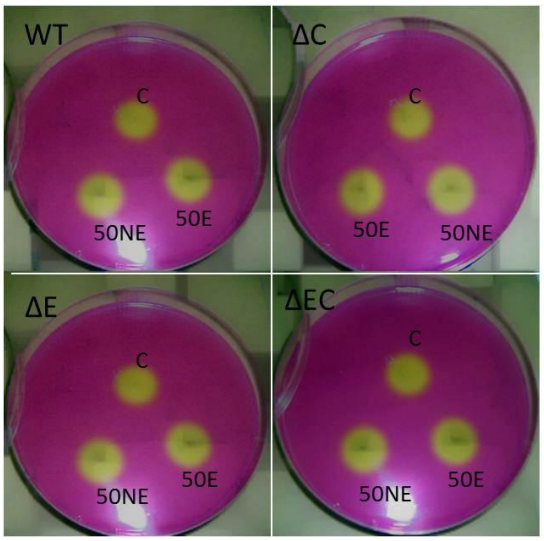


Figure 4.13 Motility of EHEC (O157:H7) 85-170 Nal^r wild-type and Δ*qseC*, Δ*qseE* and Δ*qseCE* mutants in the presence of catecholamines

A, mean data showing motility of wildtype vs mutants at Norepinephrine concentration as 5 and 50 µM (P>0.05). **B**, photograph showing a typical motility assay. WT, wildtype; ΔC, ΔqseC mutant; ΔE, ΔqseE mutant; ΔEC, Δ*qseEC* mutant.

4.3.10 Biofilm formation of EHEC wild type and Qse mutants

4.3.10.1 Biofilm formation assay

Biofilm formation of the wild type EHEC and the mutants to polystyrene in response to catecholamine exposure in DMEM and serum SAPI media are shown in Figures 4.14 and 4.15.

In DMEM medium, interestingly the *qseC* mutant biofilm formation was greater than all the other strains, including wildtype (Figure 4.14). The wildtype strain biofilm formation appeared to be enhanced by the addition of dopamine and norepinephrine but this was not statistically significant ($P>0.05$), while the *ΔqseC* mutant biofilm formation was not significantly reduced by the catecholamines dopamine and norepinephrine ($P>0.05$), but there was a significant reduction in biofilm formation in the presence of epinephrine ($P<0.05$). *ΔqseE* biofilm formation was the lowest in comparison to the other strains and the catecholamine treated culture biofilms were similar to the control cultures ($P>0.05$). However, biofilm formation was significantly enhanced in *qseCE* with norepinephrine ($P<0.05$). There was an increase in biofilm formation with dopamine, and epinephrine treatment but did not reach statistical significance ($P>0.05$).

In serum SAPI medium the biofilm formation of the strains was less compared to DMEM medium (Figure 4.15). There was no significant difference between catecholamine treated and control cultures ($P>0.05$). There was less biofilm formation observed for wildtype and *qseC* mutant compared to *qseE* and *qseEC* but again this was not statistically significant ($P>0.05$) (Figure 4.15).

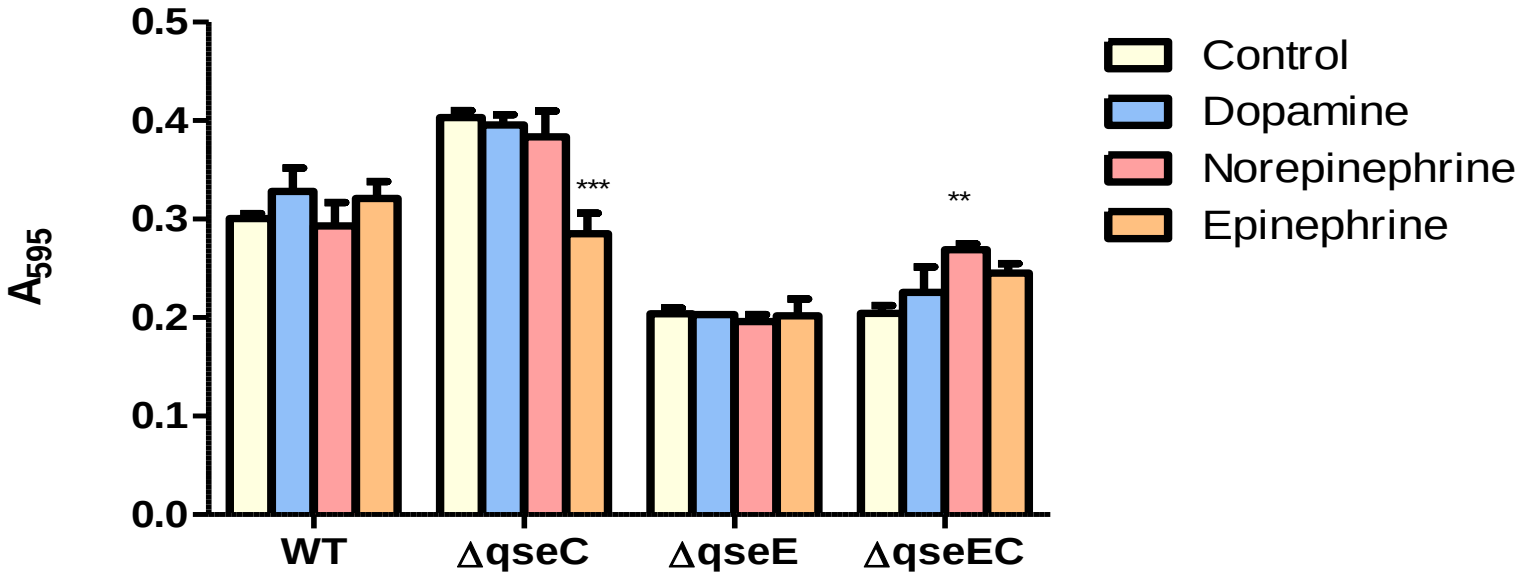


Figure 4.14 Biofilm formation of EHEC (O157:H7) 85-170 wild-type and mutants in DMEM medium.

Overnight cultures of the strains shown were diluted 1:100 into DMEM, and attachment measured following 48 hrs incubation using the crystal violet assay method. Data represent means and SEM of 4 biological replicates. Catecholamines (dopamine, norepinephrine and epinephrine) were all used at 50µM.

Keys: The symbols * indicate statistical significance of ** P<0.01; *** P<0.001

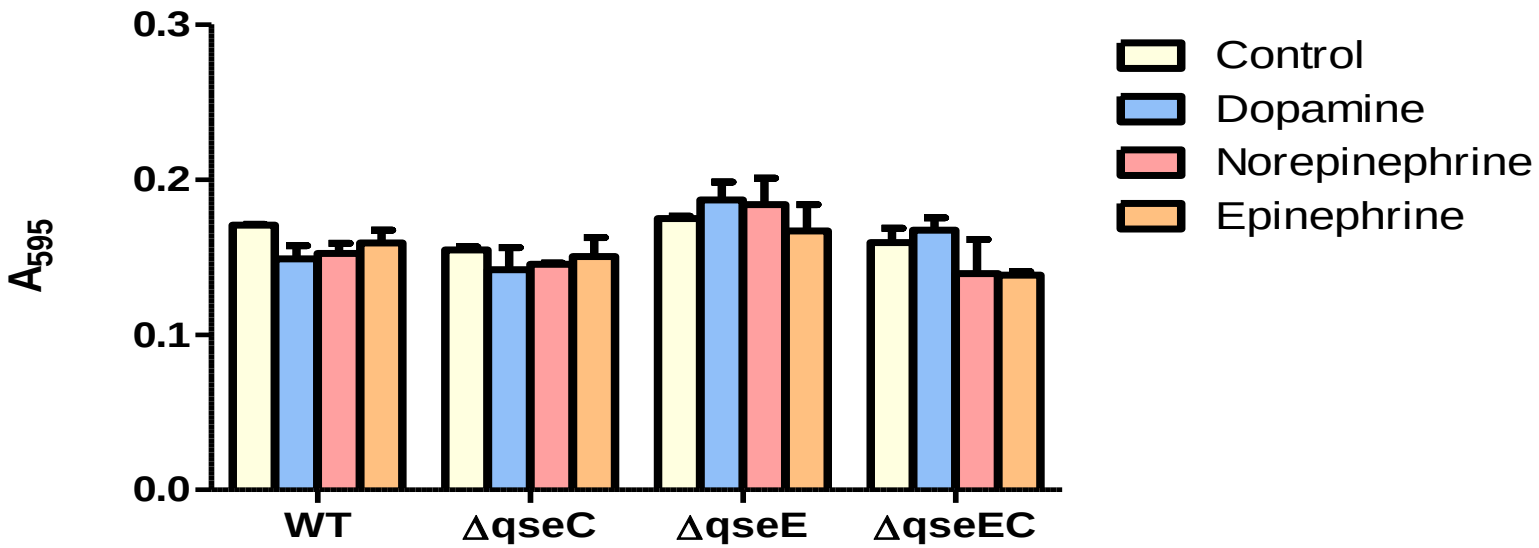


Figure 4.15 Biofilm formation of EHEC (O157:H7) 85-170 wild-type and mutants in serum-SAPI medium.

Overnight cultures of the strains shown were diluted 1:100 into serum-SAPI medium, and attachment measured following 48 hrs incubation using the crystal violet assay method. Data represent means and SEM of 4 biological replicates. Catecholamines (dopamine, norepinephrine and epinephrine) were all used at 50µM. The differences with the catecholamine treatments were not significant ($P>0.05$).

4.3.9.2 Microscopic examination of biofilm formation by EHEC and its *qse* mutants

Direct microscopic analysis of the EHEC parental and *qse* strains cultured in DMEM (Figure 4.16 A and B) showed that wildtype biofilm formation was significantly stimulated by all three catecholamines ($P<0.05$). The *ΔqseC* mutant biofilm formation was significantly increased by dopamine and norepinephrine ($P<0.001$) but significantly reduced in the presence of epinephrine ($P.<0.001$). The *ΔqseE* mutant showed the least biofilm formation of all the strains tested and was significantly reduced by any of the catecholamines ($P<0.05$). Curiously, the *ΔqseEC* mutant, which putatively is lacking both catecholamine receptors, produced less biofilm in the absence of catecholamines but was significantly stimulated by all three catecholamines ($P<0.05$) to a biofilm formation level close to wildtype.

Microscopic investigation of the mutant *qse* strain biofilm formation in serum-SAPI minimal medium (Figure 4.17 A and B) showed that the wildtype and the *qse* mutants all exhibited significant increase in biofilm formation with dopamine, norepinephrine and epinephrine ($P<0.05$). However, in this host-like medium the biofilm formation of all strains was similar. Considering Figures 4.16 and 4.17 collectively, the data strongly indicate that catecholamine stimulate biofilm formation in EHEC *E. coli* strain 85-170 and that this effect is also both influenced by the medium the bacteria are cultured in, and seems to be independent of the QseC/E gene deletion.

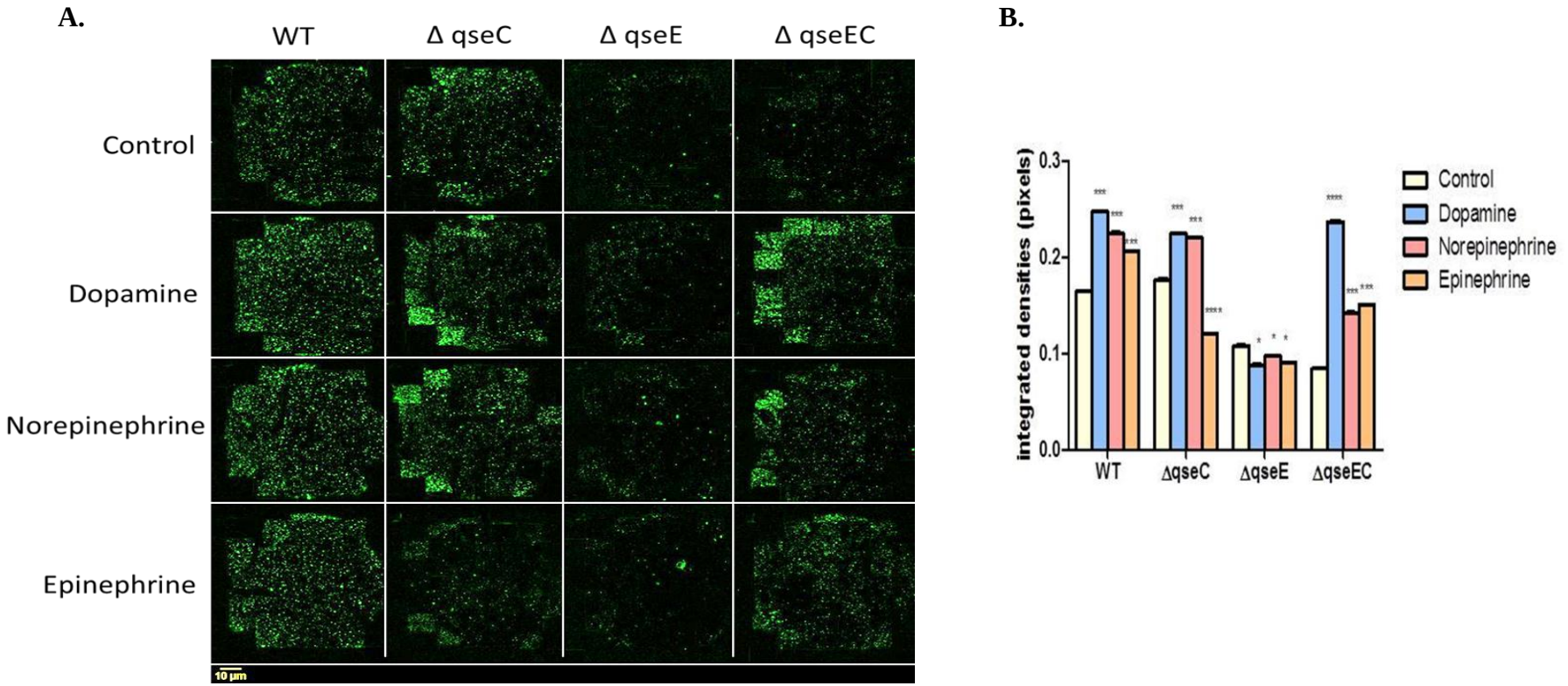
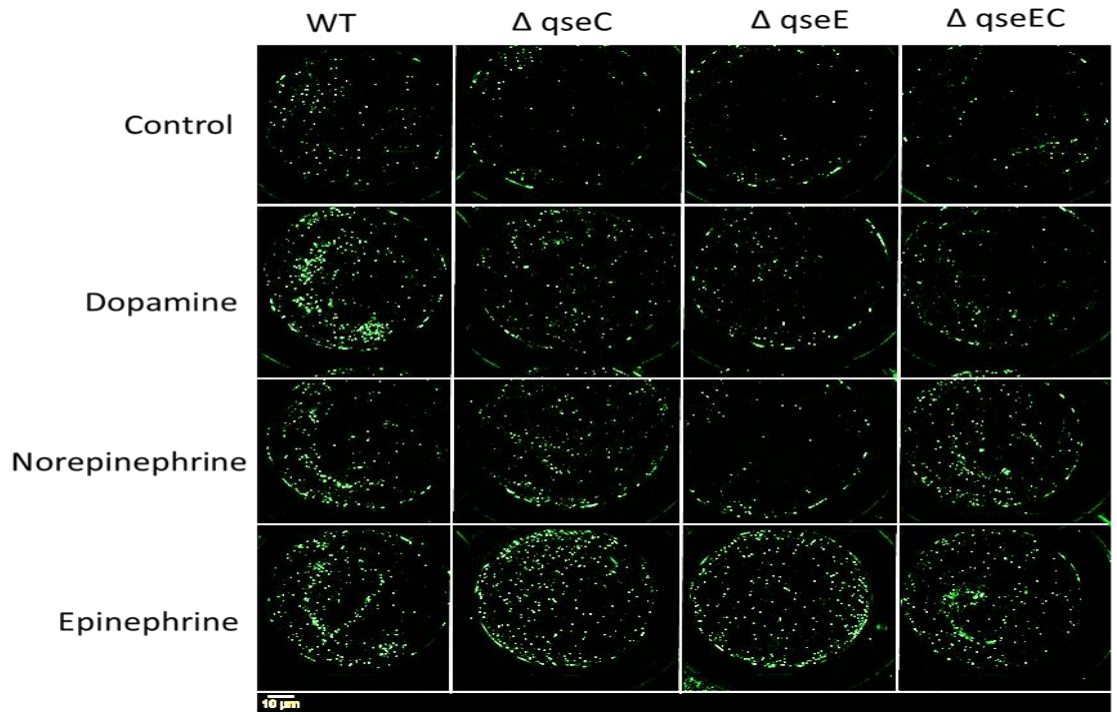


Figure 4.16 Microscopic analysis of biofilm formation by *E.coli* (EHEC) 85-170 wild type and *Δqse* mutants in DMEM.

Bacteria were incubated for 48 hrs with addition of the catecholamines dopamine, norepinephrine and epinephrine. The 96 well microtitre plate bottom were stained with biofilm live-dead stain were observed under NIKON Eclipse T inverted fluorescence microscope (10x). Biofilm formation is seen as small aggregates (green). B. Histograms showing fluorescence intensities measured as integrated densities of attached bacteria for each well. Keys: The symbols * indicate statistical significance of * P<0.05, *** P<0.001, ****P<0.0001

A.



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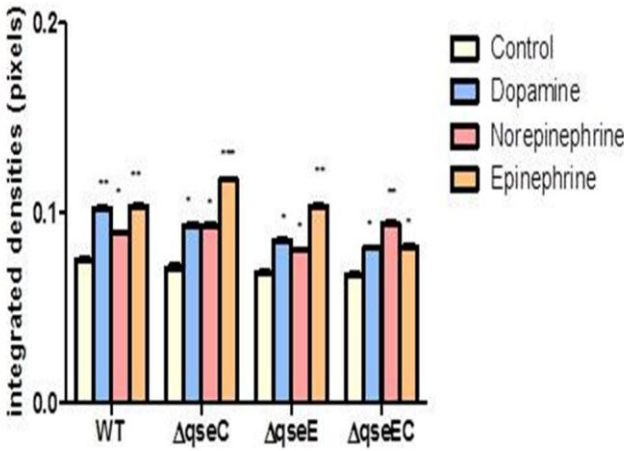


Figure 4.17 Microscopic analysis of biofilm formtion by *E. coli* (EHEC) 85-170 strain wild type and *Δqse* mutants in serum-SAPI media

Bacteria were incubated for 48 hrs with addition of catecholamines (dopamine, norepinephrine, epinephrine). The 96 microtitre plate well bottoms were stained with biofilm live-dead stain were observed under NIKON Eclipse Ti inverted fluorescence microscope (10x). Biofilm formation is seen as small aggregates (green). Hisograms showing fluorescence intensities measured as integrated densities of attached bacteria for each well. Keys: The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$; *** $P<0.001$

4.3.11 Caco-cell attachment of *E.coli* O157:H7 wild type and *qse* mutants

Attachment of enterohaemorrhagic *E. coli* to intestinal epithelium constitutes an essential step for initiation of infection (Tatsuno *et al.*, 2000). It has been shown that the *qse* mutants were defective in their ability to adhere to eukaryotic cells *in vitro* and *in vivo* (Sperandio *et al*., 2003, Clarke *et al*., 2006). Analysis of the interaction of the EHEC wildtype and strain mutants interaction with Caco-2 gut epithelial cells is shown in Figure 4.18. It can be seen that wildtype strain adherence was significantly higher ($P<0.001$) to Caco-2 cell monolayers compared to all three mutants (Figure 4.18A) but was not much affected by norepinephrine treatment.

In terms of the intracellular levels of bacteria, the wildtype, *ΔqseC*, *ΔqseE*, *ΔqseEC* were all similar in their ability to invade Caco-2 cells with and without norepinephrine. This suggests that the Qse genes may play a role in the adherence to intestinal epithelial cells, but not invasion. This effect seems to be independent of catecholamine treatment.

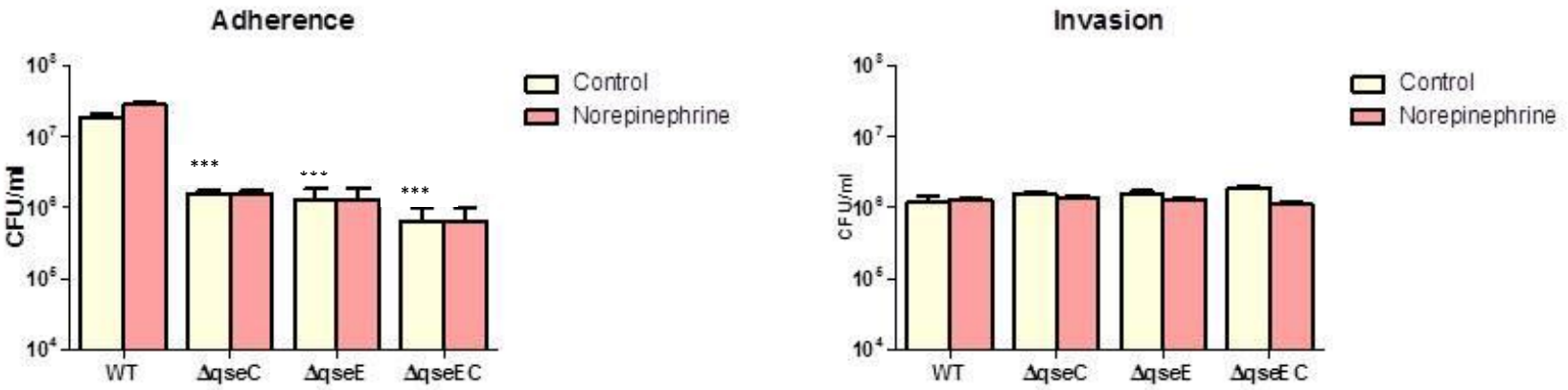
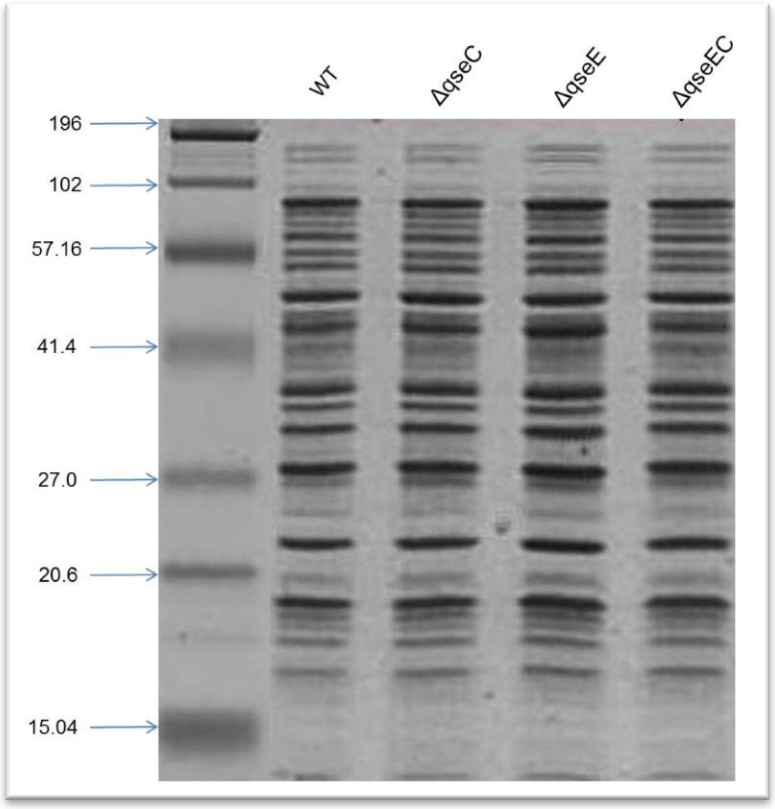


Figure 4.18 *In vitro* Caco-2 cell attachment of EHEC 85-170 wild type and *Δqse* mutants in DMEM mediaInitial inoculum was normalised to O_D600 0.1 and after 3 hrs incubation adherence and invasion was quantified as described in section 4.2.2. Histograms show a comparison of adherence and invasion ability of wildtype and *qse* mutants with and without 100 µM norepinephrine treatment. The symbols * indicate statistical significance at P<0.001*** .

4.3.12 Proteomics investigation of *E.coli* wild type and *qse* mutants

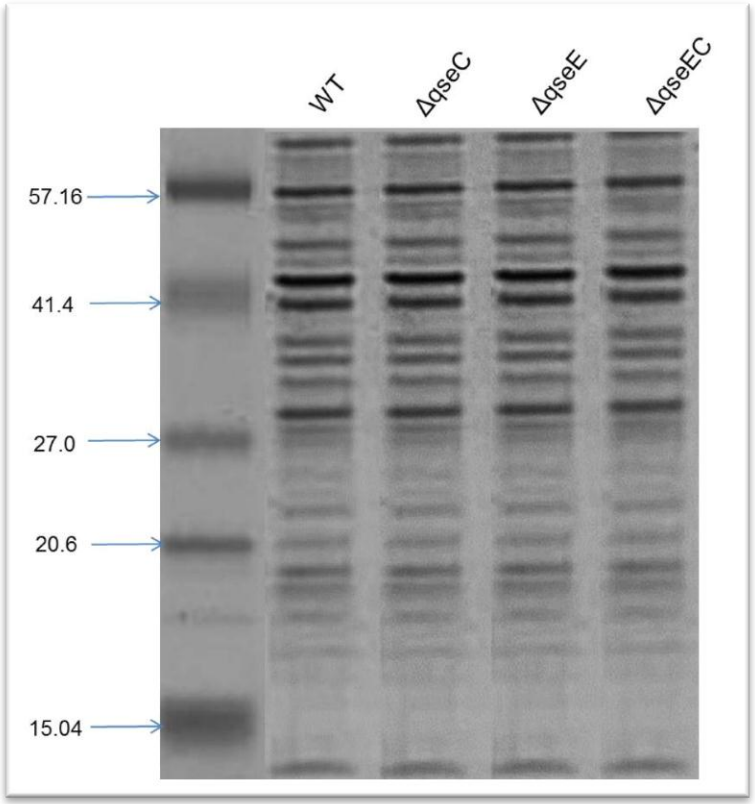
Protein expression of the wild type and the qse mutants were studied by extracting the cytoplasmic and membrane proteins and creating SDS-PAGE profiles (Figure 4.19). It can be seen that the pattern of protein expression of the wildtype and *qse* mutants were very similar.

A



Cytoplasmic proteins

B



Membrane proteins

Figure 4.19 SDS-PAGE protein profiles of EHEC wildtype and *qse* mutants. The proteins (35 µg of protein loaded per lane for cytoplasmic proteins (A) and 30µg for membrane proteins(B)) were separated on 12% [vol/vol] acrylamide gels, and stained with coomassie stain.

4.4 Discussion

It was reported that quorum sensing in EHEC regulates the expression of the locus enterocyte effacement (LEE) and senses three cell-to-cell signals (AI-3, epinephrine and norepinephrine (Sperandio *et al*., 2003) to activate expression of the LEE genes. These signals are said to be sensed by sensor kinases (QseC, QseE) in the membrane of EHEC and *S*.Typhimurium that relay this information to a complex regulatory cascade (QseB, QseF), culminating in the activation of flagella regulon, LEE, and Shiga toxin expression (Sperandio *et al*., 2003). The QseC and QseE proteins were claimed to be the bacterial adrenergic receptors for norepinephrine and epinephrine (Clarke *et al*., 2006, Reading *et al*., 2009).

A principal aim of this work was therefore to investigate whether the Qse are truly the receptors for catecholamine responsiveness in EHEC and *S*.Typhimurium. The experiments in this chapter were also carried out to investigate whether there is an effect of deleting these receptors in the formation of enteric biofilms. This is because for certain bacterial pathogens, (e.g. *P. aeruginosa S .aureus*), biofilm development is coordinated by quorum sensing (Davies *et al*., 1998). Also, it was reported that these Qse mutants were lacking motility which could also be related to biofilm forming ability of the strains since *E.coli* biofilms have been shown to be affected by motility (Pratt and Kotler, 1998).

E. coli has also been known to attach to number of artificial surfaces forming biofilms (Pratt and Kotler, 1998). In the case of uropathogenic *E.coli*, its capacity to make biofilm is one of the reasons this microbe is a major pathogen related to urinary catheter-associated infections. It was has been suggested that the QseBC two- component system controls biofilm formation in *E.coli* K-12 strain (Gonzalez *et al.*, 2006*)*. Also, EHEC microarray suggested that genes related to biofilm formation were increased following the addition of norepinephrine and epinephrine (Bansal *et al*., 2008).

The data presented in this study have investigated the effect of the eukaryotic signaling molecules epinephrine, norepinephrine and dopamine on the physiology of wild type and *qse* mutants of *E. coli* O157:H7, *S*.Typhimurium. Growth, motility, biofilm formation, protein expression, and attachment to epithelial cells were investigated.

Bacterial growth rates are influenced by multiple factors including nutrients, temperature, osmotic strength, pH, and oxygen concentration (Nackerdien *et al*., 2008). In this report in DMEM and M9 minimal medium there were found to be variations in growth of the wildtype compared with the *qse* mutants for both *E.coli* and *S.Typhimurium* (Figures 4.2, 4.11). This suggests that the *qse* gene regulation might play an important role in the metabolic activity of these bacteria. This finding supports a recent study, which has proposed that the QseBC-response system actually, may function by adjusting carbon and nitrogen metabolism to maximize general fitness during infection by pathogenic *E.coli* (Hadjifrangiskou *et al*., 2011). This suggests against the Qse proteins resembling a classical adrenergic receptor role, as in eukaryotic systems adrenergic receptors play only cell signalling roles, and are not generally responsive to cellular house keeping factors such as carbon source.

In the growth context, deletion of QseC, QseE and QseCE did not affect the ability of the *Salmonella* and *E.coli* strains to respond to NE, dopamine or epinephrine and indicates that these genes are not the receptors required for initial recognition of either adrenergic or dopaminergic catecholamines (Figure 4.3 and Figure 4.12). Also, although identical catecholamine concentrations (100 µM) were used in the growth analyses, both wildtype and qse mutant strains showed a greater preference for the host hormones they are likely to encounter in the gut (norepinephrine and dopamine) over the non-enteric catecholamine epinephrine. This specificity of catecholamine responsiveness for the hormones encountered *in vivo* is similar to that demonstrated previously for enteropathogenic *E. coli*, *Salmonella enterica* and *Yersinia enterocolitica* (Freestone *et al*., 2007a, b), and further confirms the

hypothesis by Freestone et al. (2007), that bacteria have evolved separate and specific detection systems for the host molecules they will encounter within the host niche they inhabit *in vivo*.

The motility assays of both *Salmonella* and EHEC mutants did not show significant difference with and without the addition of norepinephrine and epinephrine ($P>0.05$) (Figures 4.4 and 4.13). The *qse* gene deletion does not seem to be affecting motility as had been shown in previous studies (Sperandio *et al*., 2003, Bearson and Bearson, 2008). Merighi et al. (2010) also found there is no major role played by the *qse* genes on motility of *Salmonella* Typhimurium.

An investigation was carried out in this chapter to study the role of QseC and QseE mutation in host pathogen interaction. Previous studies have shown that the EHEC and S.Typhimurium *qse* mutants were reduced in their ability to attach to eukaryotic cell line such as HeLa cells (Sperandio *et al*., 2003, Clarke *et al*., 2006, Bearson and Bearson, 2008). This *in vitro* assay used the Caco-2 adenocarcinoma intestinal epithelial cells, which are well established as *in vitro* models for studying EHEC attachment (Izumikawa *et al*., 1998). The ability of bacteria to attach to the intestinal epithelium may help to explain the differences in pathogenicity among strains. *S*.Typhimurium invasive ability was not affected by the deletion of the QseC or QseE receptors. Merighi et al. (2009) also reported that a *qseC* mutant did not exhibit attenuation for the invasion of epithelial cells. However, the double mutant *ΔqseEC* had a significant reduction in invasion of Caco-2 cells ($P<0.01$). Adherence to epithelial cells was significantly reduced by the deletion of QseC and QseE genes ($P<0.05$). However, adherence and invasion ability was not affected by norepinephrine treatment (Figures 4.9). In contrast, the EHEC *qse* strains exhibited a difference in host epithelial cell adherence, and the *qse* mutants were found to be significantly attenuated in adherence ($P<0.001$) (Figures 4.18). Addition of norepinephrine increased adherence of *qse* mutants to Caco-2 cells and this effect

was not statistically significant (P>0.01).This finding shows that the QseC and QseE genes may play a role in intestinal adherence of EHEC, though the mechanism by which this is occurring is unclear. Another explanation might be related to reduced metabolic fitness if, as suggested the QseBC genes are involved in metabolism (Hadjifrangiskou *et al*., 2011).

The other objective of this chapter was to investigate if enteric bacteria used catechoalmines as signals to begin biofilm formation. Catecholamines did for the most part increase *E. coli* and *Salmonella* biofilm formation. However, the receptors for receiving the catecholamine signals may not be the ones proposed.

In this study, enteric pathogen biofilm formation was studied using crystal violet biofilm formation assay and direct microscopic observation using viability stains. There were differences found between *S.*Typhimurium, EHEC (O157: H7) and its *Δqse* mutants in their ability to form biofilms. These assays were conducted in DMEM and serum-SAPI media supplemented with catecholamines. DMEM medium was chosen, as it has been used in all the QseBC and QseEF two component regulation studies (Sperandio *et al*., 2003, Clarke *et al*., 2006, Bearson and Bearson, 2008). To provide a host-like environment SAPI medium supplemented with adult bovine serum was used as previously stated (Lyte and Ernst 1992, 1993, Freestone *et al*., 2008). The biofilm formation quantification (P>0.05) and microscopic investigations (P<0.05) found that there were statistically significant and non-significant differences in biofilm formation between the *Salmonella* and EHEC wild type and *qse* mutants. *S.*Typhimurium exhibited differences in biofilm formation in control and catecholamine treated cultures in both DMEM and serum-SAPI (Figures 4.5 - 4.8). Some differences in biofilm formation of the EHEC *qse* strains were also observed (Figures 4.14 - 4.17). The growth curve profiles of the wildtypes and *qse* mutants in DMEM showed marked differences (Figures 4.2, 4.11) and so the biofilm formation defects observed may be related to defects in growth and metabolism, which will affect overall fitness and so virulence. The

differences in biofilm formation of the *qse* mutants cannot be directly correlated to deletion of QSeC/E, as biofilm formation is dependent on many factors such as surface characteristics, nutrients, and other morphogenetic factors (Donlan and Costerton, 2002).

In conclusion, this *in vitro* analysis reveals that in contrast to previous studies (Sperandio *et al*., 2003, Clarke *et al*., 2006, Bearson and Bearson 2008), it is that QseC and QseE play no role in controlling *S*. Typhimurium and EHEC ability to respond to catecholamines. Collectively, this also indicates that recognition systems other than QseC and QseE may be involved in the *Salmonella* Typhimurium and EHEC O157:H7 sensing of mammalian stress hormones.

Chapter Five

Investigation of the effect of catecholamine inotropes on *Staphylococcus epidermidis* growth and biofilm formation

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5.1 Introduction

Staphylococcus epidermidis has become one of the most important pathogens in nosocomial infections especially among immunocompromised, immunosuppressed, long-term hospitalised and critically ill patients (Vuong and Otto 2002, McCann, 2008, Otto, 2009). The ability of staphylococci to colonize indwelling medical devices, such as central venous catheters (CVCs), is recognized as one of the most common source of infection encountered in the intensive care setting (Rello *et al*., 1994, Martin and Yost, 2011). Most of the catheter related infections are caused by coagulase negative staphylococci with *S. epidermidis* being the cause of 50–70% of reported cases (Rupp and Archer, 1994, Ogara and Humphreys, 2001, Götz, 2002, Mack *et al*., 2007). The pathogenicity of *S. epidermidis* is mostly due to its ability to form multi-layered biofilms on indwelling medical devices. Due to biofilm formation it has become a major problem in treating *S. epidermidis* infection as the biofilm protections make them highly resistant to antibiotics and host immune defences (more about the pathogenesis of *S.epidermidis* is discussed in section 1.4.4).

Intravascular catheters have become an integral part of complex medical and surgical procedures such as bone-marrow and organ transplantations, cancer treatment, haemodialysis, and trauma surgery leading to blood stream infections (Raad, 1998, Raad *et al*., 2002, 2007). Catheter colonisation by bacteria and other microorganisms occurs through either by the microbe colonising either the outside or the inside of the catheters. Skin commensals migrate along the external surface of the catheter and colonise the intravascular tip of the catheter, whereas, hub contamination leads to luminal colonisation and bloodstream infection after reaching a threshold number of organisms about 15-1000 bacteria (Sitges-Serra and Girvent, 1997, Raad, 1998, Bayston and Fisher, 2009). A wide variety of catheters and infusion sites

are used to deliver drugs, supportive and nutrition fluids to seriously ill patients. Peripheral veins are most commonly used for general access; while the central veins such as the subclavian are used for total parenteral nutrition (Marie and Costerton, 1984, Cook *et al*., 2007) Intra-arterial catheters are also used to monitor arterial pressure (Marie and Costerton, 1984).

Changes in environmental conditions are known to affect staphylococcal biofilm formation (Lyte *et al*., 2003, Fitzpatrick *et al*., 2005). One such effector might be related to drug exposure. Surveys of ICU drug usage indicate that critically ill patients typically receive multiple daily medications of more than 20 different compounds in the course of an ICU stay (Smyth *et al.*, 1993), usually administered by intravenous infusion at very high doses with up to half of these patients receiving catecholamine inotropes (dopamine, epinephrine, norepinephrine, dobutamine, isoprenaline) (Freestone *et al*., 2010). Previous work has demonstrated that medications administered through central venous catheters (CVCs), such as the catecholamine inotropes (norepinephrine, epinephrine, dopamine and dobutamine) which are used to maintain cardiac and renal function, can promote *S. epidermidis* growth in the planktonic state (Neal *et al*., 2001). In the case of norepinephrine and dobutamine, an ability to stimulate biofilm formation on intravenous catheter material (Lyte *et al*., 2003) was also observed. In host-like media supplemented with serum and plasma, it has also been demonstrated that antibiotic-damaged *S. epidermidis* were recovered after exposure to catecholamine inotropes (Freestone *et al.*, 2008).

The main objective of this chapter was to determine if all catecholamine inotropes were signals to stimulate *S. epidermidis* to make biofilm, and to undertake a phenotypic analysis to

understand the effect of catecholamine inotropes on staphylococcal biofilm formation in different environmental conditions.

5.2 Specific Methods

Ethics statements: Human blood from which plasma was prepared was obtained from healthy volunteers. Consent was obtained from all individuals, who were not taking anticoagulant therapy, according to the protocol for blood donation approved and in compliance with the research ethics of University of Leicester.

5.2.1 Culture conditions

The bacterial strain used in this study was the biofilm-producing *S. epidermidis* RP62A (ATCC, 35984). Heparin used in this study was kindly provided by Dr.Mohammed Yusuf & Eman Abu-rish, University of Leicester. In previous studies catecholamine inotrope effect on *S epidermidis* attachment to surfaces, was studied in platelet rich plasma SAPI (Lyte *et al.*, 2003), and so this medium was used in some of the studies in this chapter.

Blood samples were collected from volunteers, and transferred to collecting tubes containing heparin (1 µl/ml of blood sample). Heparinised blood was then centrifuged at 1500 x *g* for 20 min to give platelet-rich plasma; this was prepared fresh for each experiment and was diluted one in ten in SAPI medium and used immediately.

The catecholamine inotropes concentration (100 µM) used in this study is based on the previous studies (Neal *et al*., 2001, Lyte *et al*., 2003). TSB medium was iron restricted by adding 300 µM dipyrydyl as described previously (Wise *et al*., 2002).

5.2.2 Haemagglutination Assays

The hemagglutination assay can be used as an indirect assay for the PIA/PNAG expressed by *S. epidermidis* strains, as it has been shown to be responsible for their ability to agglutinate red blood cells (Mack *et al*., 1999, Joyce *et al*., 2003). The haemagglutination activity of *S. epidermidis* was assessed using the method previously described (Rupp and Archer, 1992, Rupp *et al*. 1995). Bacteria from an overnight incubation in Trypticase Soy Broth (TSB) were grown in plasma-SAPI at 37°C, harvested by centrifugation and washed once with PBS. The bacterial suspensions were adjusted to an OD_{600} approximately of 1 (approximately 10^8 CFU/ml), and then diluted 1:10 in PBS. The hemagglutination assay was performed using 96-well (U-shaped) microtitre plates). Twofold serial dilutions of the 10% bacterial suspension were performed in the microtitre plates to give a total volume of 50 µl per well. To prepare the red blood cells, 10 ml sheep blood was centrifuged at 1500 x g for 15 minutes and diluted in PBS to obtain a 1% suspension; 50 µl of the 1% sheep erythrocyte suspension was then added to each well. The plates were shaken gently for the even mixing of the bacteria and erythrocytes, and then incubated at room temperature for 2 hrs. To assess hemagglutination of biofilm bacteria, the cultures were grown in plasma SAPI medium in petri dishes for 24 h at 37°C. The supernatant was removed, and the cells were scraped from the surface into 10 ml of phosphate-buffered saline. After passage through a 23-gauge needle the bacterial suspension was adjusted to an OD_{600} of 1.0 (Mack *et al*., 1999). Haemagglutination assay was performed as described above for planktonic bacteria.

All tests were performed in triplicate. A positive result was defined as the production of diffuse red blood cells with no red blood cells pelleting at the bottom of the well.

5.2.3 Haemolysis Assays

Haemolytic assay was performed by the microplate method of Sato et al. (Saito et al., 2006; Cheung *et al*., 2010) using sheep erythrocytes. In these experiments, 10 ml of sheep blood was centrifuged at 1500 x g for 15 minutes and then diluted in PBS to obtain a 4% suspension. A culture supernatant of overnight-grown bacteria at 37°C in serum SAPI medium was used for haemolytic assays. Two-fold dilutions of the culture supernatant in PBS (pH 7.0) were mixed with 50 µl of 4% RBC in PBS in a 96-well microtitre plate (U-shaped). The microtiter plate was incubated at 37°C for 2 h after gentle shaking. 10% SDS with erythrocytes was used as a positive control. The microtitre plate was centrifuged at 300 x g and the supernatant was removed and transferred to a new microtitre 96 well plate, absorbance was read at 540 nm in ELISA reader. The plates were visually read and the haemolytic titre was calculated as the highest dilution at which haemolysis could be detected.

5.2.4 Total cellular protein analysis of planktonic and biofilm bacteria

Overnight optical density-normalised cultures of *S.epidermidis* RP62A adjusted to OD600~ 0.1 were used. The diluted culture (0.5 ml) was then used to inoculate 50 ml of plasma SAPI medium in polystyrene petri dishes (140mm diameter, triple vent, Sterilin, Newport, UK) to give a final population density of ca. 10^6 CFU/ml. Cultures were incubated with and without catecholamine inotropes at 5 and 100 µM concentrations (Dopamine, NE, Epi); all cultures were then incubated at 37°C in a 5% CO_2 incubator. After 24 h planktonic bacteria were removed, the plates were washed with ice cold PBS six times to remove the non-adherent bacteria and the biofilms were detached by scraping the petri dish with a cell scraper. The biofilm cultures were harvested by centrifugation for 10 min at 12, 000 x g at 4°C. The bacterial pellets were resuspended in PBS, containing 0.3 mg of phenylmethylsulfonyl

fluoride/ml, and cells were lysed by sonication (5 treatments for 10 s at 4 W, maintaining a temperature of 4°C). Cell debris and unbroken cells were removed by centrifugation at 10000 rpm, for 10 minutes. Total protein concentration was quantified, and the cell protein samples processed for SDS PAGE as described in section 2.5.

5.3 Results

5.3.1 Catecholamine inotropes increase *S. epidermidis* growth

Incubation of an inoculum of around 100 CFU/ml *S.epidermidis* in plasma-SAPI for 18 hrs with the inotropes norepinephrine, dopamine, epinephrine, at 100 µM resulted in an up to 100 -fold significant increase in bacterial numbers over the un-supplemented control cultures ($P<0.001$ for both strains) (Figure 5.1). Longer incubations (48 hrs, right panel) showed that control and inotrope cultures had reached a similar cell density. At a higher inoculum 10^5 CFU/ml the growth of test and control cultures was similar (data not shown).

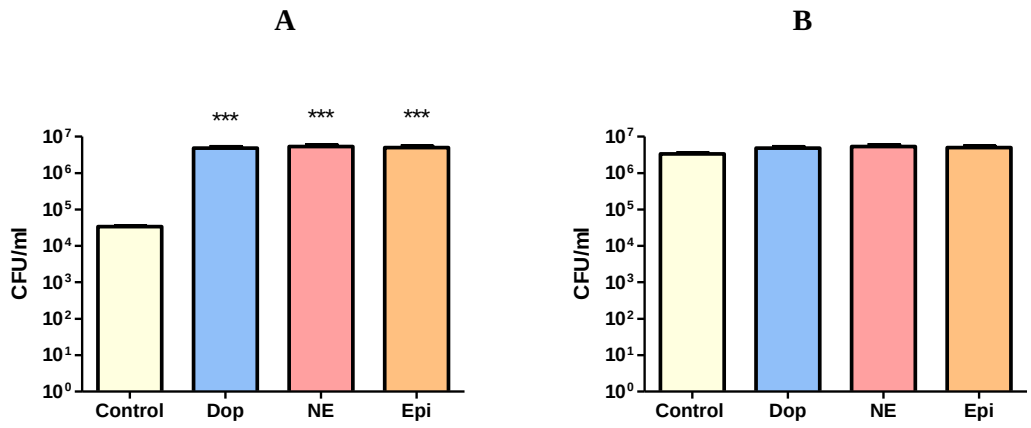


Figure 5.1 Effect of catecholamines on growth of *S. epidermidis* RP62A. Overnight cultures were diluted in 10-fold steps to a <100 CFU/ml as shown on the x axes into plasma SAPI with or without the catecholamines (all at 100 µM), grown for 24h statically at 37°C. Bacterial growth levels were enumerated by serial dilution of the cultures followed by plating onto LA. Values represent the means and standard deviations of triplicate platings from triplicate experimental cultures (n=3). The first panel A represents growth in 24 hrs and the second panel B represents growth in 48 hrs. Keys: Dopamine (Dop), Norepinephrine (NE), Epinephrine (Epi). The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$; *** $P<0.001$.

5.3.2 Catecholamine inotropic agents increase biofilm formationof *S. epidermidis* in plasma SAPI medium

The biofilm formation of *S. epidermidis* was studied using the Crystal violet microtitre biofilm formation assay as described in section 2.2.3.3. Different inoculum sizes were used to investigate whether the biofilm formation was growth related as catecholamine growth responsiveness has shown to be occurring when the inoculum size was less than 100 CFU/ml (Freestone and Lyte, 2009). As shown in figure 5.2 biofilm formation of *S. epidermidis* to polystyrene was significantly increased when a higher *S. epidermidis* inoculum was used, ($P<0.001$). In contrast the biofilm formation level of the cultures was apparently lesser at lower initial inoculum sizes. The intropes tested (dopamine, norepinephrine and epinephrine) all significantly increased biofilm formation ($P<0.001$) at the higher inoculum, while at a low inoculum size (100 CFU/ml) only dopamine significantly increased biofilm formation compared to the control ($P<0.05$).

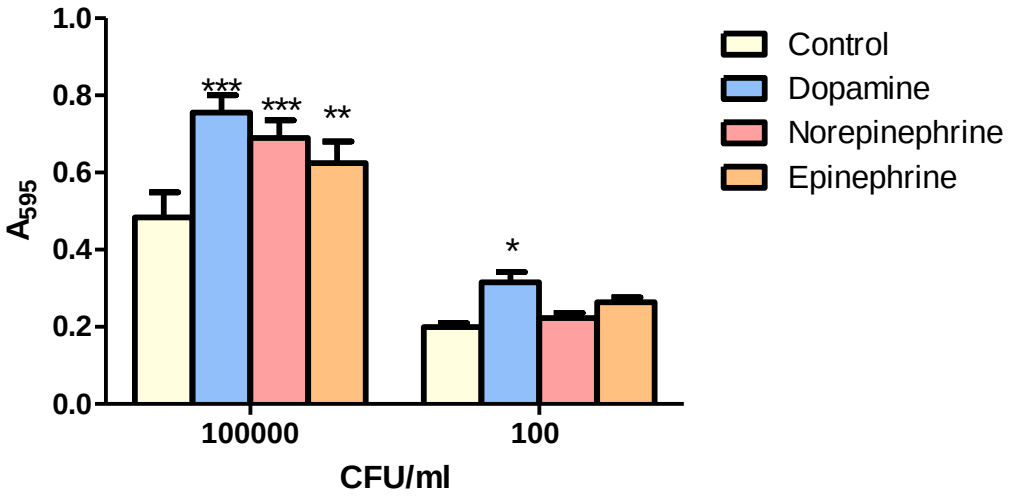


Figure 5.2 Inotrope on biofilm formation of *S.epidermidis* (RP62A) to a polystyrene surface in host-like medium. Overnight cultures were diluted 1:100 into plasmaSAPI media, and biofilm formation measured after 48hrs incubation. Data shown represent means and SD of 4 biological replicates from triplicate assays. Key: The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$*** $P<0.001$.

5.3.3 Inotropes effects on *S.epidermidis* biofilm formation in nutrient rich medium

To investigate if inotrope effects on biofilm formation was dependent on factors within serum or plasma *S. epidermidis* was grown in tryptone soy broth supplemented with inotropes for 24 hrs and an microtitre plate biofilm formation assay was carried out as described in section 2.2.3.3. The TSB medium was also made iron deplete by the addition of dipyridyl, to mimic at least one condition that is host like (Ratledge and Dover, 2000). Figure 5.3 shows that there was a significant increase in biofilm formation with catecholamine inotrope treatment ($P<0.05$). However, in iron depleted conditions there was not much difference in the biofilm formation levels with or without the inotropes which was not statistically significant ($P>0.05$). This result suggests that catecholamine induced-biofilm formation is not media-dependent and in nutrient rich condition the stimulation is not iron-dependent.

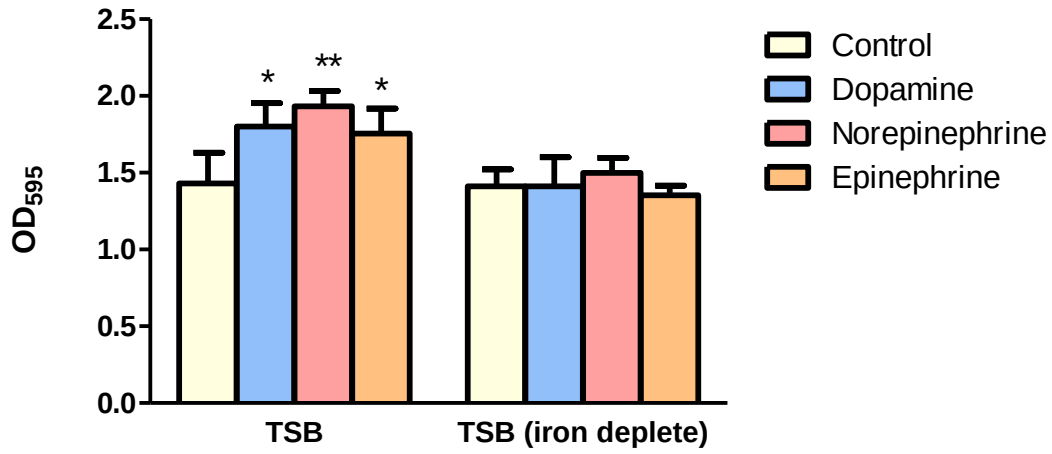


Figure 5.3 Inotrope on biofilm formation of *S.epidermidis* on a polystyrene surface in nutrient rich medium. Overnight cultures were diluted to 10^5 cells/ml into TSB medium only and TSB iron depleted with 300 µM dipyridyl, and biofilm formation using the crystal violet method measured after 24 hrs of incubation. Data shown represent means and SD of 4 biological replicates from triplicate assays. Key: The symbols * indicate statistical significance of * $P<0.05$, ** $P<0.01$.

5.3.4 Catecholamine inotropes enhance *S.epidermidis* biofilm formation on intravenous catheters

Polyurethane intravenous catheters were cut into small pieces (1cm) and inoculated with *S.epidermidis*, (10^5 CFU/ml) in test media with inotropes (as described in the individual experiments) and incubated for 48 h and processed for Scanning Electron Microscopy as described in section 2.2.4 (Figure 5.4). As shown in Figure 5.5 compared to the un-supplemented control cultures, the inotropes (dopamine, norepinephrine and epinephrine) all induced bacterial attachment, colonisation and biofilm formation (n=3) in host-like plasma-SAPI medium. Interestingly, the outer surface of the catheters appeared to have less biofilm formation than the inner lumen surface. Also, the inoculum size used in the catheter biofilm experiments (10^5 CFU/ml), meant that the control and inotrope-treated cultures always showed similar rates of growth and final cell densities (data not shown). This suggests that the catecholamine biofilm formation on the intravenous catheters surface was not a growth-related effect, as already discussed in section 5.3.2. EPS fibres within the biofilms were not prominent in the SEMs in Figure 5.5.

SEM investigations of biofilm formation on the catheter surface of *S.epidermidis* RP26A grown in plasma SAPI medium sometimes showed the presence of flaky deposits spread over both outer and inner surface irrespective of catecholamine treatment, and *S.epidermidis* colonies were observed embedded in these deposits. Comparing the inner and outer surfaces of the IV catheter section, there was differences in the appearance of these accretions (Figure 5.6). The inner surface had more densely packed deposits whereas, the outer surface showed loosely spread deposits. These deposits or amorphous substances are most probably plasma proteins as these deposits were not observed in biofilms formation in the presence of nutrient rich medium (data not shown).

The SEM analysis of RP26A biofilm formation on catheters incubated in nutrient rich medium (TSB) showed the catheter surface was covered with a bacterial monolayer and in some areas a high degree of biofilm formation was also observed (Figure 5.7). On the inner side of the catheter it can be seen that biofilm formation was occurring for both the control and inotrope treated cultures, and that the addition of the inotropes did not produce much difference. Interestingly, the outer surface of the catheter showed prominent mushroom-like biofilm structures with catecholamine treatment (which are not observed *in vivo*), while the unsupplemented control did not show these mature biofilm structures (Figure 5.7). Signs of EPS production was much prominent (seen as fibres) in the images in Figure 5.7. In both host like and laboratory medium it can be seen that the inotropes significantly stimulated more biofilm formation compared to control cultures.

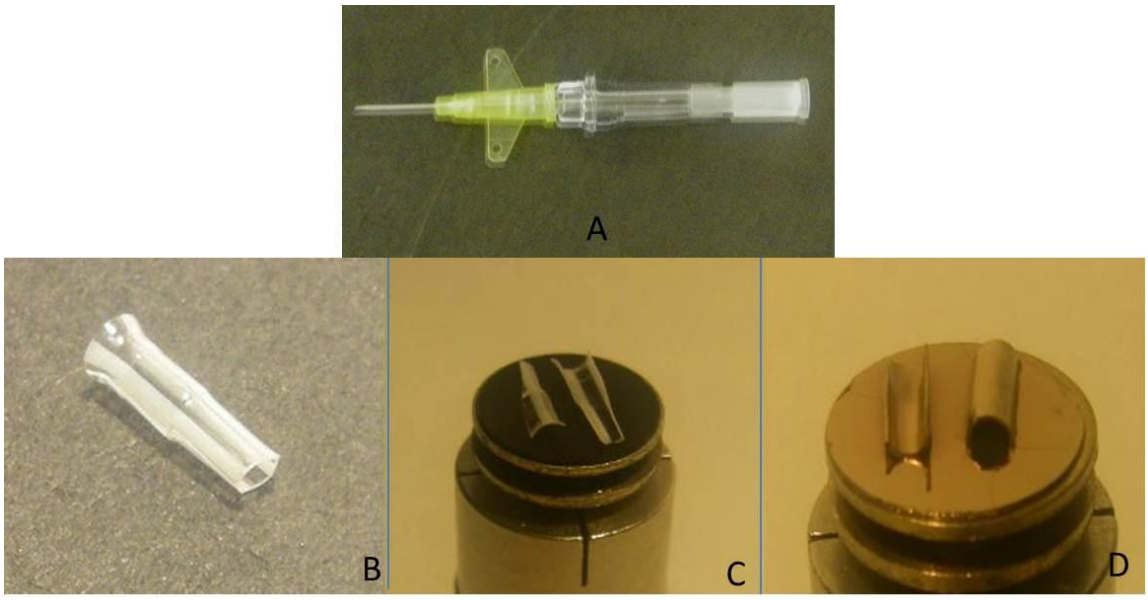


Figure 5.4 Images of the intravenous catheter sections: Panels showing polyurethane catheter sections before and after processing for scanning electron microscopy. A. An intravenous catheter; B, the section used; C, catheter cut into halves showing outer and inner surfacesl; D, Gold sputter coated catheters used in SEM showing inner and outer surface.

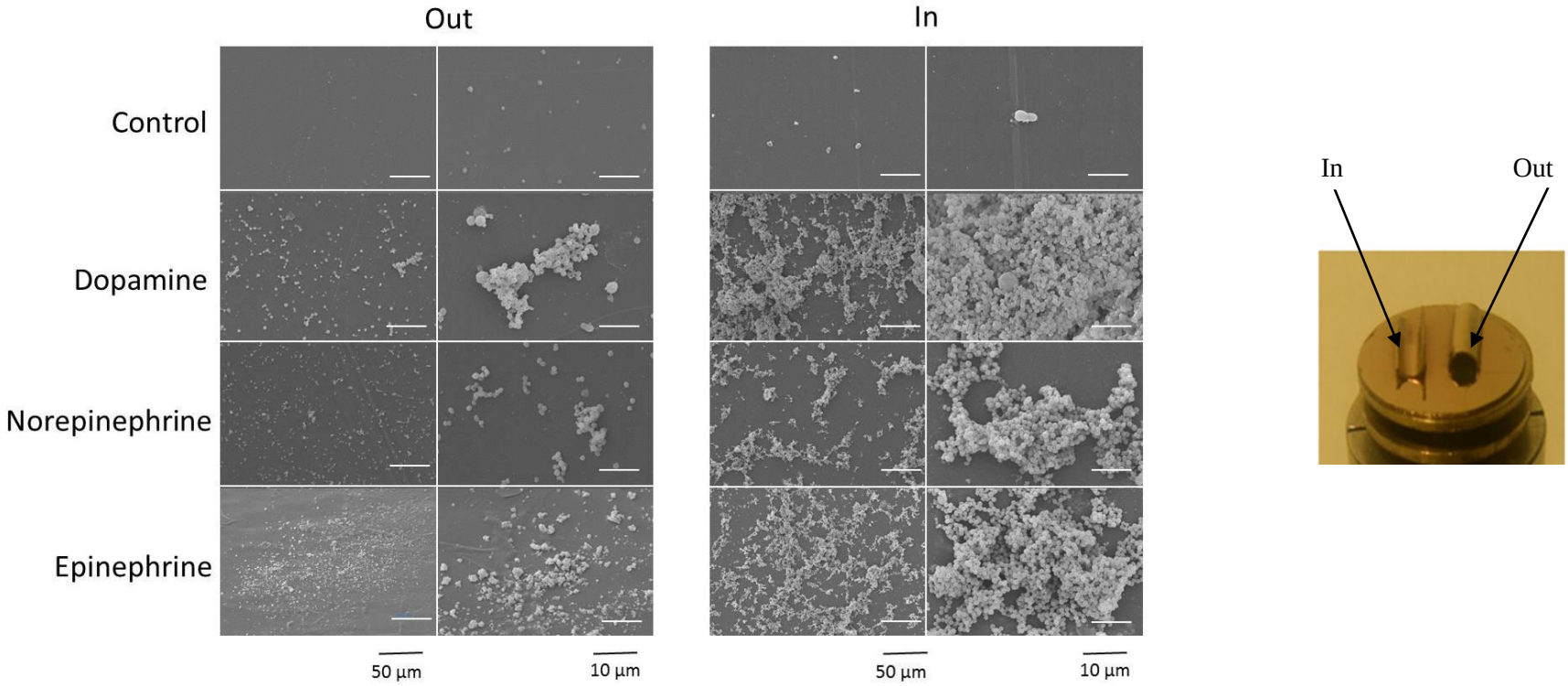


Figure 5.5 Scanning electron microscope images of catecholamine inotrope effects on *S.epidermidis* biofilm formation on intravenous catheters. The panel of images show SEMs of catecholamine inotrope-enhancement of *S.epidermidis* biofilm formation on polyurethane catheter material. *S.epidermidis* RP26A was inoculated at $2x10^5$ CFU/ml onto sterile catheter sections in the absence or presence of norepinephrine (100 µM), epinephrine (100 µM) and dopamine (100 µM) in plasma-SAPI medium and incubated for 48 hrs as described in section 2.2.3.4. The 2 sets of panels show representative SEMs (of 3 separate experimental sets) of the catheter outer and inner surfaces at increasing magnification; the scales for each image are shown in the bottom of the figure.

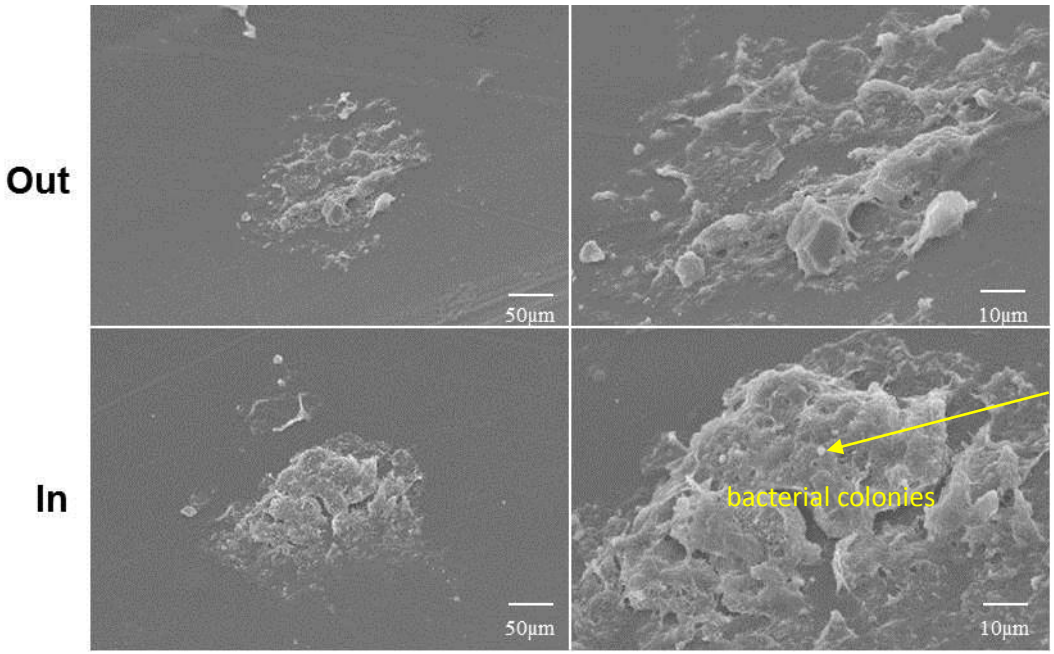


Figure 5.6 Scanning electron microscopic (SEM) images of the outer and inner surface of the polyurethane catheters showing flaky deposits in host-like medium, Culture of the inner surface of and outer surface of intravenous catheter. Note presence of bacterial colonies embedded in the deposits

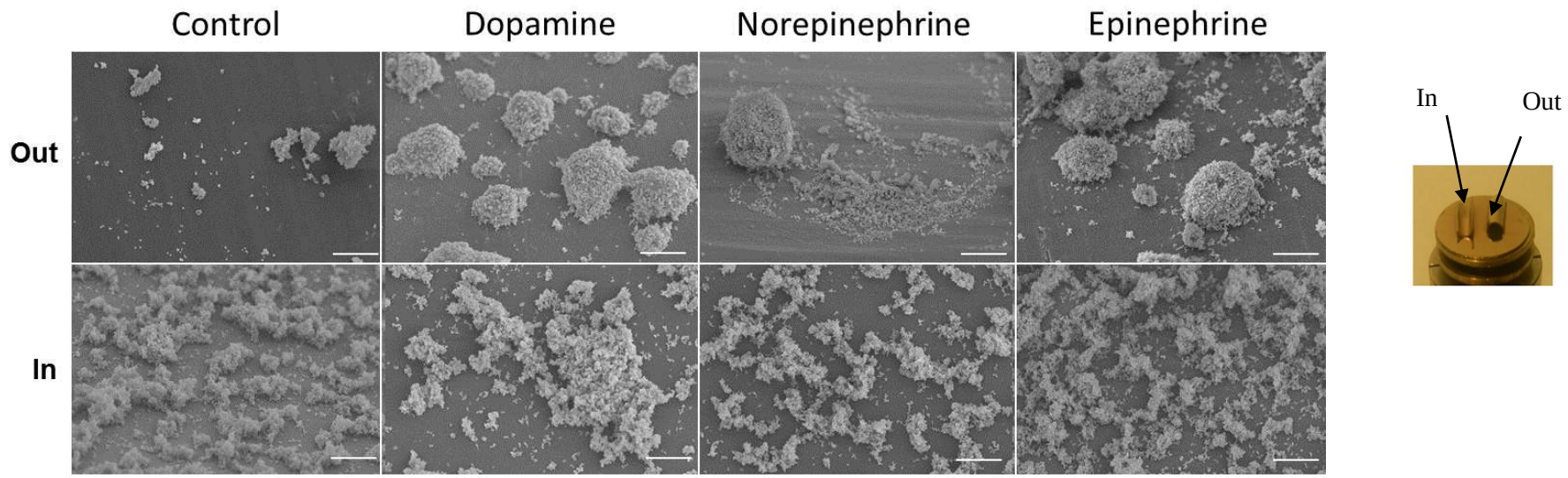


Figure 5.7 Scanning electron microscope images of catecholamine inotrope effects on *S.epidermidis* RP26A biofilm formation on intravenous catheters in nutrient rich medium. The panel of images show SEMs of catecholamine inotrope effects of *S.epidermidis* biofilm formation on polyurethane catheter material. *S.epidermidis* was inoculated at $2x10^5$ CFU/ml onto sterile catheter sections (1 cm) in the absence or presence of norepinephrine, epinephrine and dopamine (all 100 µM) in TSB medium and incubated for 24 hrs as described in Materials and Methods 2.2.3.4 (n=3). The 2 sets of panels show representative SEMs (of 3 separate experimental sets) of the catheter outer and inner surfaces, the scale is 50 µm.

5.3.5 Effect of catecholamine inotropes on extracellular proteins in *S.epidermidis* biofilms matrix

Biofilms of *S. epidermidis* RP62A grown in plasma SAPI in the presence and absence of catecholamine inotropes were stained with the red fluorescent protein dye SYPRO Ruby in order to visualize extracellular proteins among biofilm matrix. It can be seen that a large amount of extracellular proteins were visible in biofilms grown in the presence of inotropes (Figure 5.8). This result shows that catecholamine inotropes stimulate extracellular matrix proteins which is important for biofilm development.

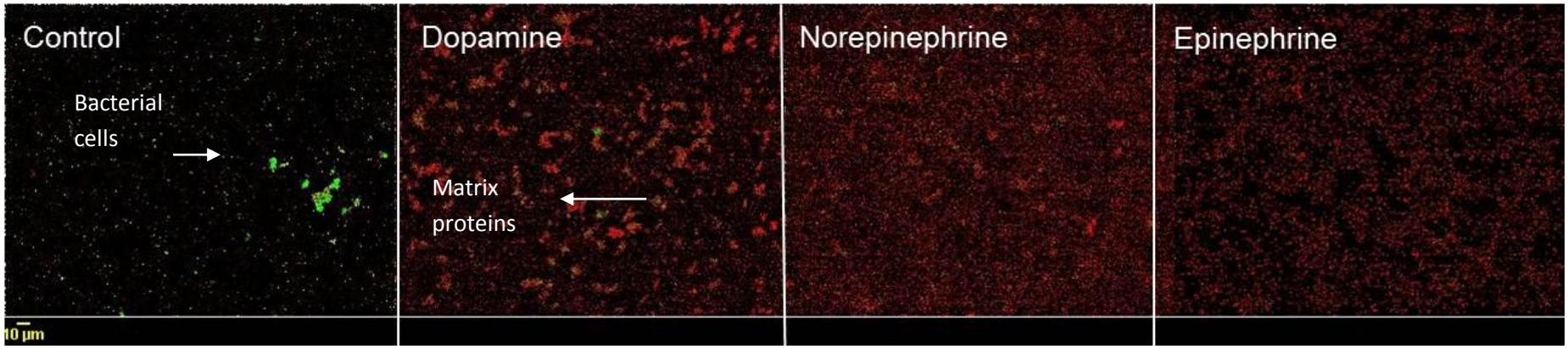


Figure 5.8 Detection of extracellular proteins in biofilm matrix of *S. epidermidis* RP62A.

48 hrs grown biofilms in plasma SAPI medium with no addition (Control) and addition dopamine, norepinephrine and epinephrine (100 µM) were stained with SYPRO Ruby matrix stain (red) and Syto 9 stain (green) visualised under NIKON Ti 2000 inverted florescent microscope under 40x objective at 465/610 excitation and emission spectra. Green colour: bacteria cells embedded in the matrix Red deposits- biofilm matrix proteins

5.3.6 Inotropic agent effects on *S.epidermidis* haemagglutination

The ability of *S. epidermidis* to mediate hemagglutination of erythrocytes of different species (degree of hemagglutination was different with different species as shown in the studies of Rupp *et al*., 1995,1999, Mack *et al*., 1999) has been shown to be associated with the ability to adhere to plastic and to produce biofilm and therefore may be important for the pathogenesis of S. *epidermidis* infections (Rupp *et al*., 1995,1999, Mack *et al*., 1999). An investigation was carried out to understand whether the catecholamine inotropes (Dop, NE, Epi) had any effect on hemagglutination activity of *S.epidermidis* cultured in plasma. Figure 5.9 shows that there was no hemagglutination observed in the presence or absence of catechomine inotropes in host like medium. The biofilm bacteria too showed similar results to the planktonic bacteria (data not shown)

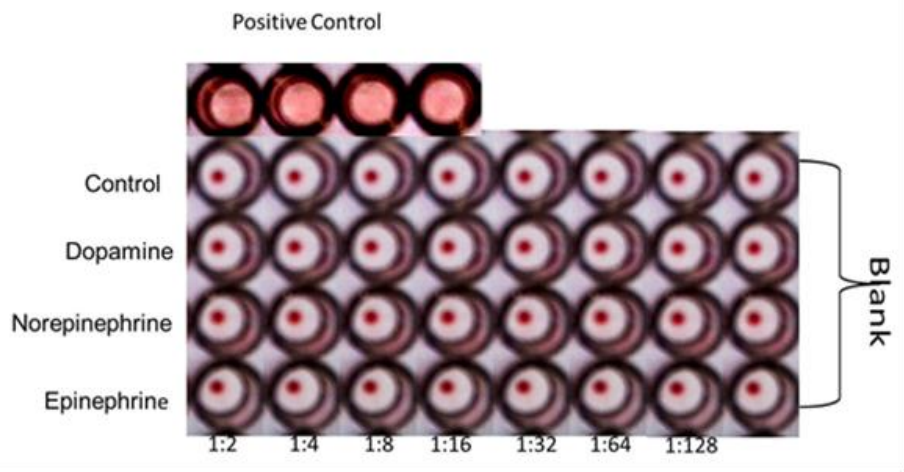


Figure 5.9 Effect of catecholamine inotropes on *S.epidermidis* haemagglutination of sheep erythrocytes by *S. epidermidis* RP26A.

The cultures were grown in plasma SAPI at 37 °C for 20 hrs and concentrated tenfold (5 x 10^{7} CFU/ml) prior to performing the haemagglutination assay. The positive control was *S. epidermidis* RP26A grown in Tryptone soy broth. Bacterial dilutions are shown at left, ranging from 1:1 to 1:128. The positive control shows the production of diffuse red blood cells, while the negative control (**Blank** set of well on the figure) appears as as no red blood cells pelleting at the bottom of the well.

5.3.7 Catecholamine inotrope effects on *S.epidermidis* haemolysis of erythrocytes

Production of haemolysins is one of the principal pathogenicity factors of *S. epidermidis*. Lysis of red blood cells is primarily mediated by the haemolysin delta (δ) toxins (Vyong and Otto, 2002, Michellim *et al*., 2005). An investigation was carried out to determine whether catecholamine inotropes stimulated the haemolytic activity of *S. epidermidis*. As shown in Figure 5.10 while there was some lysis of the red blood cells, there was no significant difference ($P>0.05$) in the haemolytic titre of the culture supernatants of *S. epidermidis* following culture with the catecholamine inotropes. Haemolytic assays were also carried out in nutrient rich TSB medium, which also showed similar results (data not shown). These findings show that catecholamine inotropes apparently do not play a role in stimulating production of *S. epidermidis* haemolysins.

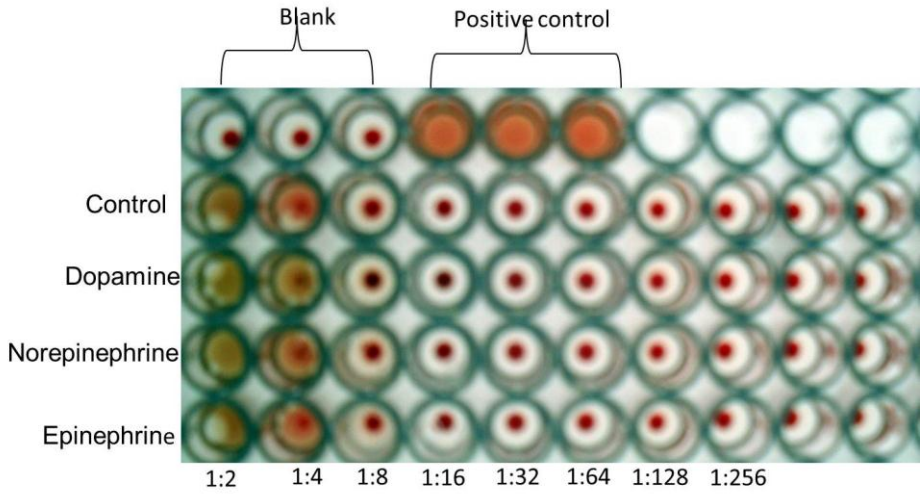


Figure 5.10 Effect of catecholamine inotropes in the production of haemolysins of *S.epidermidis* RP26A:

Haemolysis assays were carried out as described in section 5.2.3. Key: No haemolysis (Blank), and Positive Control (10% SDS). Representative results are shown and assays in duplicate were carried out at least three times.

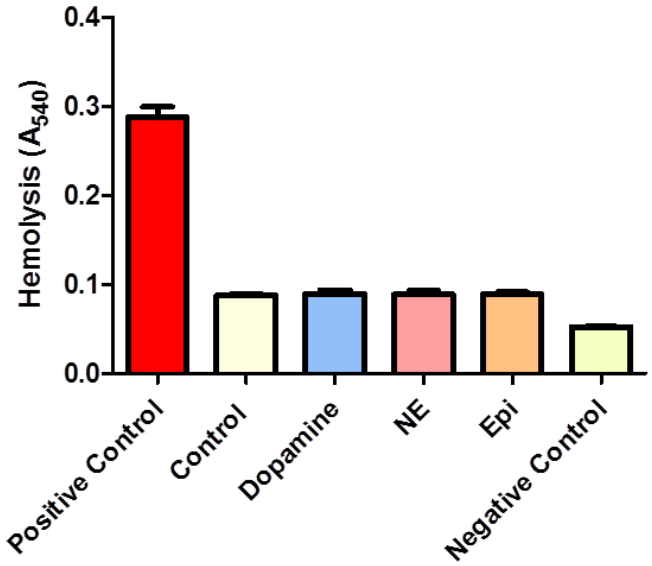


Figure 5.11 Haemolysis quantitative assay using sheep blood.

The data show haemolysis titre quantified after measuring the supernatant absorbance at 540 nm. Negative control, PBS; positive control,10% SDS in PBS.

5.3.8 Catecholamine inotrope effect on *S.epidermidis* protein expression

Whole cell proteins were extracted from planktonic and biofilm bacteria as described in section 5.2.4 and 2.4. As show in Figure 5.12, there were differences between planktonic and biofilm protein expression. However, there were no obvious differences in protein expression found between the inotrope treatment and non-supplemented control cultures.

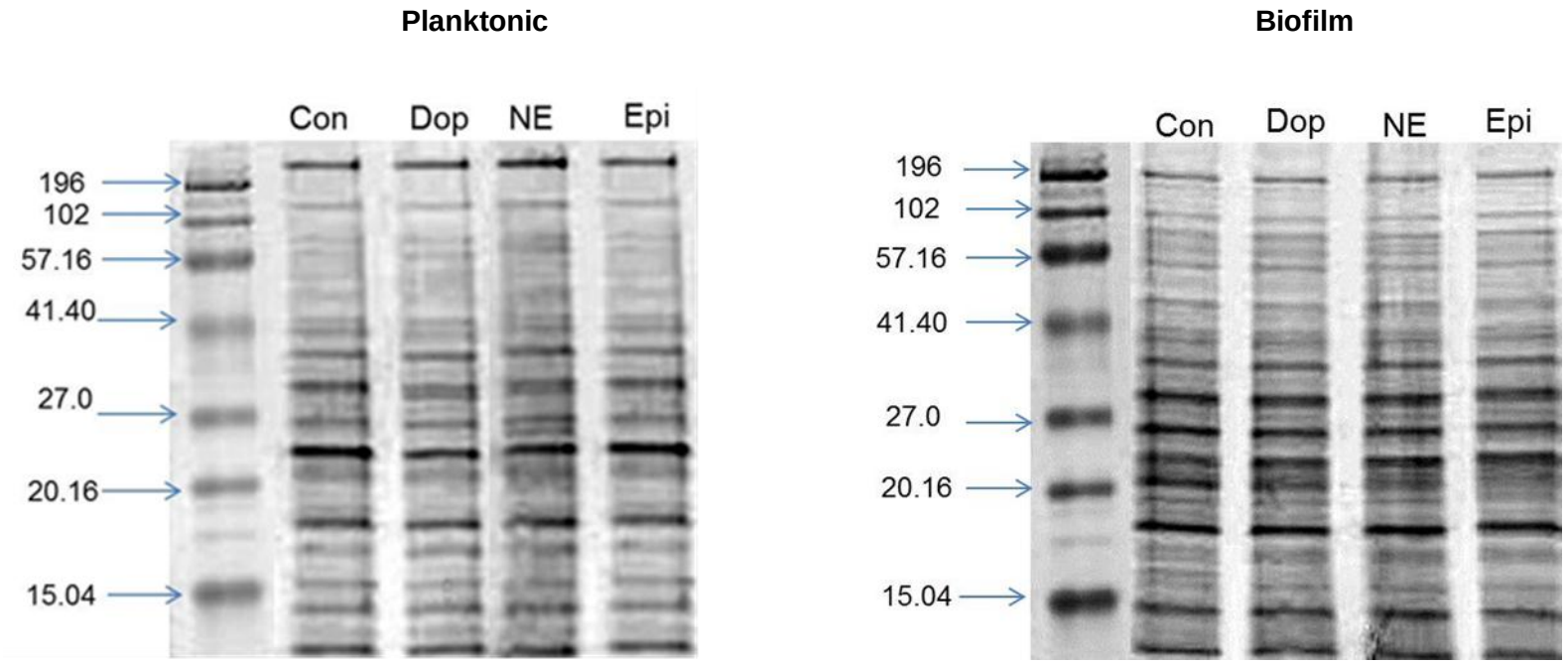


Figure 5.12 SDS-PAGE whole cell protein profiles of *S.epidermidis* Bacteria were grown in TSB medium in the presence of 100 µM of the catecholamines shown (Dop, NE and Epi) and planktonic and biofilm (24 h) proteins were extracted as described in section 5.2.6. The proteins (25 µg of protein loaded per lane) were separated on 12% [vol/vol] acrylamide gels, and stained with coomassie brilliant blue stain. Representative gels are shown which are typical of three separate experiments. Keys: Con- Control, Dop-Dopamine, NE- Norepinephrine, Epi- Epinephrine.

5.4 Discussion

S. epidermidis biofilm formation has been proposed to occur in a two-step manner which involves initial attachment of bacterial cells to a surface, followed temporally by cell multiplication and formation of a mature structure in a matrix of associated multiple layers (Mack *et al*., 1999). Staphylococcal biofilm formation often occurs in response to signals present in the surrounding environment and a variety of factors are known to stimulate biofilm formation (Fitzpatrick *et al*., 2002, 2005). In a previous study two catecholamine inotropes used in the intensive-care setting (1000 μM Dobutamine and 100 μM norepinephrine) were both shown to stimulate *S epidermidis* biofilms on silicone and polystyrene catheter plastics. The process was found to occur in the presence of human plasma and involved the acquisition by bacteria of iron from the serum protein transferrin (Lyte *et al*., 2003). The current investigation was carried out to understand if other intropes administered via intravenous catheters (dopamine and epinephrine) and media-related factors also affected *S. epidermidis* biofilm formation.

In the Lyte et al. study, only the catecholamines dobutamine and norepinephrine were investigated; this study differs from this investigation in that it used a wider variety of the inotropes that are commonly infused through intravenous catheters, and used concentrations of less than 1mM. In plasma-supplemented media the catecholamines tested (dopamine, norepinephrine and epinephrine) all enhanced biofilm formation of *S. epidermidis*. Also, this chapter found that catecholamine-induced biofilm formation was not dependent on bacterial growth rates (Figure 5.2). However, planktonic growth stimulation was observed only at less than 100 cfu/ml in plasma supplemented medium (Figure 5.1) and in serum SAPI (Neal *et al*., 2001). Previous studies have shown that the catecholamine-induced growth mechanism in

S.epidermidis involves iron acquisition from transferrin in iron restricted plasma and serum-supplemented media (Neal *et al*., 2001; Lyte, *et al*., 2003, Freestone *et al*, 2008).

A previous finding has shown that catecholamine hormones at 50 µM concentration (dopamine, norepinephrine and dopamine) were able to induce *S. epidermidis* RP62A attachment in nutrient rich media such as TSB (Frank and Patel, 2008). This current study has also showed that catecholamine inotropes stimulate significant biofilm formation ($P<0.05$) in nutrient rich laboratory medium. However, in more host-like iron depleted media conditions there were no differences in biofilm formation (Figure 5.3). Sandrini et al. (2010) showed that in liquid media catecholamines can directly complex ferric iron; since Fe limitation can induce staphylococcal biofilm formation (Deighton and Borland 1993, Baldassarri *et al*. 2001) this may explain why inotropes stimulated biofilm formation in iron replete TSB but not in media that had made already iron depleted by the addition of the Fe chelator dipyridyl. Strains of *S. epidermidis* which were initially biofilm negative were induced to form biofilms when grown in iron-starved medium (Deighton and Borland 1993), and iron limitation has been shown to induce slime production by some staphylococci strains (Baldassarri *et al*., 2001).

The SEM images of *S epidermidis* biofilm formation on intravenous catheter material (Figures 5.4 - 5.7) showed that the catecholamine inotropes stimulated biofilm formation on both the inner and outer surfaces significantly compared to the un-supplemented control which had very few colonies of surface attached bacteria. This result confirms the previous finding (Lyte *et al*., 2003) which showed that norepinephrine and the synthetic catecholamine dobutamine increased biofilm formation of *S.epidermidis* on polystyrene and silicone catheter material. In the current study the polyurethane catheter surface biofilm formation was a little

less compared to the Lyte *et al*. study. However, Lyte *et al*. (2003) performed their experiments in a shaking CO_2 incubator which has been shown to facilitate more biofilm formation (Biswas and Biswas, 2005). This study also extends the range of inotrope effects on *S epidermidis* biofilm formation to also include dopamine and epinephrine. In the current study inotropes all markedly increased staphylococcal biofilm formation on polyurethane polymers incubated under static culture conditions (Figure 5.5).

The EPS production of the catecholamine-stimulated biofilms was not that prominent in the host-like medium in this study compared to the previous report by Lyte *et al*. (2003). This may be because in the previous work Critical Point Drying (CPD) was used to process the biofilms for SEM and in this study hexamethyldisilazane (HMDS) treatment was performed in sample processing for SEM (Lyte *et al*., 2003). Use of CPD has shown to be preserving EPS better than HMDS treatment (Ratnayake *et al*., 2012).

It was also found that biofilm formation on polyurethane catheter sections varied between the inner and out surfaces of the catheter section, with more occurring within the lumen in plasma SAPI medium (Figure 5.5). In the nutrient rich medium three dimensional biofilms were seen on the outer surface compared to the inner surface did not have these projections (Figure 5.7). This may happen since the outer surface is rougher than the inner surface, a rough surface has a greater surface area and the depressions in the roughened surfaces provide more favourable sites for colonization (Scheuerman *et al*., 1998, Katsikogianni and Missirlis, 2004).

In the first *S epidermidis* inotrope study Lyte et *al*., (2003) found that the presence of catecholamine inotropes increased production of PIA (polysaccharide intercellular adhesin)

which is a major component of the *S. epidermidis* biofilm matrix. Extracellular proteins of a considerable variety (environmental and bacterial) are also an important component of the bacterial biofilm matrix and contribute to adhesion and overall biofilm formation processes (Sousa *et al*., 2008, Otto, 2009). Interestingly, the results of this current study demonstrate that catecholamines not only stimulated production of the exopolysaccharide component of the matrix but also increased biofilm levels of the matrix proteins (Figure 5.8). Mack and colleagues (1999) have demonstrated that there are quantitative and functional relationships between PIA produced, and the haemagglutinin of *S. epidermidis*. Even though, PIA production was increased when exposed to the inotropes (Lyte *et al*., 2003) in this study in host-like plasma-SAPI medium *S. epidermidis* did not haemagglutinate erythrocytes with or without catecholamine inotropes. This lack of haemagglutination may be a strain-related effect or nutrient effect, and its absence did not seem to affect biofilm attachment or formation.

Haemolytic activity has been detected in some *S. epidermidis* strains, α-hemolysin having been associated with neurotoxic activity and γ-haemolysin with a severe inflammatory response (Michelim *et al*., 2004). The production of phenol soluble modulins (PSMs) that are not potent cytotoxins would thus ascertain that *S. epidermidis* may cause chronic, biofilm-associated infection without promoting acute, purulent inflammation (Cheung *et al*., 2010). This study showed *S.epidermidis* RP26A haemolytic titre was not significantly affected by catecholamine inotrope treatment($P>0.05$).

An aim of this chapter was to investigate whether there are any differences in protein expression of planktonic and biofilm bacteria. Understanding of the expression of proteins involved in catecholamine induced biofilm formation will be very useful in identifying

different treatment strategies in vascular catheter related infections. However, there was a problem obtaining a reasonable protein yield due to difficulty in obtaining sufficient amount of plasma for culturing, as these experiments require minimum 100 ml of the plasma supplemented medium carried out in duplicate at least three times. EDTA anticoagulated plasma inhibited *S.epidermidis* attachment and only fresh heparinised plasma was useful. The pooled plasma from different donors did not induce good attachment of the bacteria and the *S.epidermidis* attachment ability was found to be markedly influenced by gender, age, diet etc (data not shown). This latter finding also shows that there are person-variable factors in the human host which might also contribute to bacterial biofilm formation. Therefore, only certain donors were selected on the basis of the results of the biofilm formation and for ethical reasons these donors could only be approached on a limited number of occasions. However, the proteomics experiments were also conducted in TSB medium as it had been showed that inotropes significantly increased biofilm formation ($P<0.001$) on polystyrene and more biofilm on polyurethane IV sections (Figure 5.3 and 5.7) However, the catecholamine inotrope treatment did not produce much differences in protein expression (Figure 5.11) using either one dimensional gel electrophoresis (Figure 5.11) or even 2-D¬PAGE (data not shown) . A feasible *in vitro* experimental design for *S.epidermidis* catecholamine responsiveness has to be developed in order to carryout molecular biological analysis.

This study confirms the previous finding that catecholamine inotropes are signals which induce biofilm formation of *S. epidermidis*. It also demonstrates new findings that other inotropes such as dopamine and epinephrine can also stimulate biofilm formation in both host-like and nutrient rich laboratory media.

CHAPTER SIX

General Discussion

General discussion

Health care associated infections, one of the leading causes of disease world wide, are a major health issue (CDC, 2011). Infected medical devices are a common source of nosocomial infections and contribute to substantial morbidity and mortality (WHO, 2011). The most significant hospital-acquired infections, based on incidence and potential severity, are those related to surgical site infections and medical devices (Bayston, 2000, Guggenbichler *et al.*, 2011). At least half of all cases of nosocomial infections are associated with medical devices including pneumonia in patients intubated on a ventilator and bacteraemia related to intravascular catheter use. (Richard *et al*., 1999, Safdar *et al*., 2001, Vincent, 2003, Lyte *et al*., 2003).

Many environmental factors and intrinsic biological properties influence biofilm formation and its subsequent development and behaviour (Bayston, 2000, Sauer and Camper, 2001, Donlan, 2002). These environmental factors now also include human hormones and drugs. A correlation between stress and infection of human and other animals has long been established (reviewed in Freestone *et al*., 2008). Released during stress are a variety of chemical signals, principal of which are the catecholamines (Reiche *et al.*, 2004). As well as their effects on immune cell responses, catecholamine stress hormones and structurally similar drugs have all been shown to stimulate bacterial growth and virulence (discussed in section 1.3) Lyte et al. (2003) showed that catecholamine stress hormones (epinephrine and NE) and the structurally similar synthetic drug dobutamine not only accelerated planktonic growth of *S. epidermidis*, but they also enhanced biofilm formation on catheter-grade plastics (Lyte *et al*., 2003). This discovery of drug-related stimulation of biofilm formation (Lyte et al, 2003) started the research which has led to the *P. aeruginosa*-VAP paper of Freestone et

al. (2012), and the suggestion that drugs which are used in the hospital setting may serve as environmental factors leading to bacterial biofilm formation of indwelling medical devices.

The research in this thesis was conducted on the hypothesis that catecholamine hormones as inotropes are a new class of environmental signals which lead to stimulation of biofilm formation of certain infectious bacteria on indwelling medical devices. The findings and significance of each chapter are summarised below.

6.1 Catecholamine inotropes effect on *P. aeruginosa*

In Chapter three, it was found that catecholamine inotropes used in the ICU setting can stimulate growth and biofilm formation of both clinical strain and laboratory strains of *P. aeruginosa*. The mechanism by which inotropes increased *P. aeruginosa* growth involved the inotrope enabling the bacteria to access the iron within transferrin coupled with the direct internalisation of the catecholamine and allows better iron removal by *P. aeruginosa* siderophore pyoverdine. The Fur (Ferric iron uptake regulator) protein was found to be present only in the inotrope-treated cultures which further confirms the above finding that catecholamine inotropes aid in iron acquisition. Interestingly the outer membrane protein OprF identified in the inotrope-treated culture has now been identified as the transferrin binding receptor in *P. aerugi*nosa (Dr. Freestone personal communication)

Another important finding was that inotropes most frequently administered to ventilated patients (norepinephrine, epinephrine and dopamine), all markedly increased *P. aeruginosa* biofilm formation and subsequent development on endotracheal tubes. This discovery as bacterial colonisation of the ET is a factor thought to influence ventilated patients to development of pneumonia (Adair *et al.*, 1999, Alp and Voss, 2006, Pneumatikos *et al.*, 2009). Interestingly, this study also showed that catecholamine inotropes also affected factors

relevant to biofilm formation such as flagellar and twitching motility, microcolony formation and EPS production. This further suggests that there could be causal connection between inotrope medication and pseudomonal biofilm formation.

Another important finding of this study was that significant stimulatory effects of inotropes on *P. aeruginosa* pathogenicity could be achieved using clinically relevant drug concentrations ($P<0.05$) (Thompson *et al*., 1999, Girbes *et al*., 2000), which has direct relevance for the the capacity of *P. aeruginosa* to cause ventilator-associated pneumonia. The non-catecholamine inotropes vasopressin and phenylephrine were in contrast to the catecholamines, non-stimulatory to either to the growth and virulence of *P. aeruginosa* strains tested. However, a full and comprehensive analysis of the effects of vasopressin and phenylephrine on *P. aeruginosa* is needed before it can be said that they have no influence at all on the bacteria.

The study in chapter 3 in conclusion suggests that administration of inotropes to patients in intensive care, particularly if high doses are given systemically or via direct local application, may be a risk factor in the development of ventilator associated pneumonia by *P. aeruginosa*.

6.2 Effects of catecholamines on biofilm formation by Enteric bacteria

Recently, there has been a growing interest in the deadly pathogens EHEC, *Salmonella*, *Shigella* and other food-borne pathogens, in understanding the pathogenic mechanisms of these organisms due to their clinical and epidemiological significance (Bansal *et al*., 2007). During infection in the gastrointestinal tract, pathogens such as enterohaemorrhagic *E. coli*O157:H7 and *Salmonella* may be exposed to a wide range of signaling molecules, including the eukaryotic hormones. The catecholamine hormones have been shown to play an important role in the pathogenesis of enteric bacteria and adhesion and infection of the

above bacteria (see section 1.3.3- 1.3.5). The AI-3/NE/Epi signal-dependent QSeBC and QseEF have been proposed to play an important role in the attachment and motility of EHEC and S. Typhimurium. QseC and QseE proteins were also proposed to be the adrenergic receptors for norepinephrine and epinephrine (described in section 4.1). Rasko et al. (2008) have proposed that inhibition of interkingdom intercellular signalling constitutes an effective strategy for the development of a novel set of antimicrobial agents and Qse genes are been proposed for drug targets to treat EHEC related infections (Rasko *et al*.,2010). However, in the last 2 years since this claim no evidence has been presented relating to QSe-specific drugs, which in light of the recent finding that QseBC are involved in metabolism (Hadjifrangiskou *et al.*, 2011), suggests that more research is needed into the roles of the Qse gene products before any highly expensive future drug developments take place.

As explained in chapter four, the phenotypic comparison of EHEC and *S*.Typhmurium wild types and their respective *qseC qseE* and *qseEC* mutants did not show significant differences in relation to growth, motility ($P>0.05$). Differences in biofilm formation were statistically significant ($P<0.05$) or did not reach statistical significance ($P>0.05$) with catecholamine stress hormone treatment in different media. However, there was a significant reduction of adherence to intestinal epithelial cells by the *qse* mutants of both enteric species ($P<0.05$). Differences were also observed in the mutants' growth patterns in laboratory nutrient rich and poor media (section 4.3), which as stated above would agree with the proposed role of the Qse pathway in metabolism (Hadjifrangiskou *et al*., 2011). This study also found that QseC, QseE and QseEC mutation play no role in controlling *Salmonella* Typhimurium and EHEC ability to respond to catecholamines and to form biofilms. This suggests that the proposed adrenergic bacterial receptors are not needed for sensing the catecholamines. There may therefore be other catecholamine hormones sensing systems involved in the two organisms tested.

6.3 Effects of catecholamine inotropes on *S.epidermidis* biofilm formation

Vascular accesses are an essential tool of modern medicine (Guggenbichler *et al*., 2011) In recent years and in correlating with increased usage of invasive medical procedures, the more opportunistic pathogen coagulase-negative staphylococci (CoNS), and in particular *S. epidermidis,* have become one of the most important risk factor of nosocomial infections (Huebner and Goldman 1999, Geffers *et al*., 2003, Bayston *et al*., 2009). *S. epidermidis,* have become recognized as serious nosocomial pathogens associated with indwelling medical devices such as catheters and prosthetic valves (Mc Cann *et a*l., 2008)

Previously, Lyte and co-workers (2003) showed that catecholamine inotropes such as dobutamine and norepinephrine stimulated growth and biofilm formation of *S. epidermidis* in a host-like medium on clinical grade silicone and polystyrene plastic. The findings of chapter five confirm this previous finding and also further show that other catecholamine inotropes such as dopamine and epinephrine can also induce *S. epidermidis* biofilm formation. It was also found that inotrope-induce biofilm formation was not dependent on bacterial inoculum size. However, catecholamine growth responsiveness was observed only in low inoculum sizes (10-100 CFU/ml). Another finding of this study was that the catecholamine biofilm stimulation occurred even in the laboratory nutrient rich media, and that media iron depletion did not have any influence in the inotrope effect as it was already in the host like nutrient poor medium. Biofilm extracellular protein production was also stimulated in host-like medium by the inotropes. Another, interesting finding was polyurethane catheter colonisation and biofilm formation was markedly increased with inotrope treatment. This study shows that all catecholamine-type inotropes can act as signals to stimulate *S.*

epidermidis biofilm formation. Clinically, the therapeutic relevance of this work is similar to that of the Lyte et al, 2003 study – catecholamine inotropes could promote *S. epidermidis*-related infections on indwelling catheters by stimulating biofilm formation.

Future work

Analysis of catecholamine effects on bacterial biofilm formation on different clinical plastic would be interesting. Also, visualising inotrope effects in real time using time lapse analysis using flow cell systems with confocal microscopy would be useful in understanding the role of catecholamine stress hormones and inotropes at each stage of biofilm development on different biomaterials.

Two dimensional gel electrophoresis-based proteomic analysis and protein microarray analysis over time also needs to be carried out to identify the proteins and genes involved in catecholamine-induced biofilm formation in the different stages of biofilm development. Such investigations would give a global picture of what happens when the catecholamine meets the bacteria, and could target genes for alternative therapeutic strategies for biofilm associated infections such as VAP and intravascular catheter related infections.

Antimicrobial agents are sometimes incorporated into the polymers of indwelling medical devices such as catheters and ET tube. However, Freestone *et al,* (2008) showed that norepinephrine and dopamine can both assist staphylococcal species to recover from antibiotic-damage. Interestingly, the same study also showed that adrenergic and dopaminergic antagonists such as phentolamine and chloropromazine could block bacterial growth responses to the inotropes, which also prevented their resuscitation from the antibiotic challenge. It is therefore possible that incorporating these antagonists into the polymers used for manufacture of the indwelling plastic might, along with an antimicrobial agent, reduce inotrope effects on any bacteria present.

Further, investigations are also needed to determine whether the circulating catecholamines of inotrope-medicated ICU patient combined with the presence of naturally occurring catecholamines in blood and mucosal secretions, are high enough to promote growth and virulence of *P. aeruginosa* and *S. epidermidis* associated with any medical devices.

Conclusions

The work presented in this thesis has fulfilled the main objectives of the study which were to investigate *in vitro* whether catecholamine stress hormones and inotropes were acting as novel environmental signals to promote the biofilm formation of infectious bacteria.

From the clinical perspective, this study suggests that administration of inotropes to patients in intensive care, particularly if high doses are given systemically or via direct local application via catheters or endotracheal tubes, may be a risk factor for the development of ventilator associated pneumonia by *P. aeruginosa* and catheter-associated blood stream infections by *S. epidermidis*.

A final suggestion for the future is that it might become necessary is to extend the patient side effect information on drug labels to also include effects on the microbes inhabiting the patient!

Appendix I

Papers published during the course of my PhD

Freestone, P. P., Hirst, R. A., Sandrini, S. M., Sharaff, F. F., Fry, H., Hyman, S., and O'Callaghan (2012). *Pseudomonas aeruginosa*-catecholamine inotrope interactions: A contributory factor in the development of ventilator associated pneumonia?

Chest published May 3, 2012, doi:10.1378/chest.11-2614

Sharaff F and Freestone P. (2011): Microbial Endocrinology. *Central European Journal of Biology*, 6(5):685-694.

Pullinger, G. D., Carnell, S. C., Sharaff, F. F., van Diemen, P. M., Dziva, F., Morgan, E., Lyte, M., Freestone, P. P. E., Stevens, M. P. (2010) Norepinephrine Augments *Salmonella enterica*-Induced Enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. *Infection and Immunity*, 78(1):372–380.

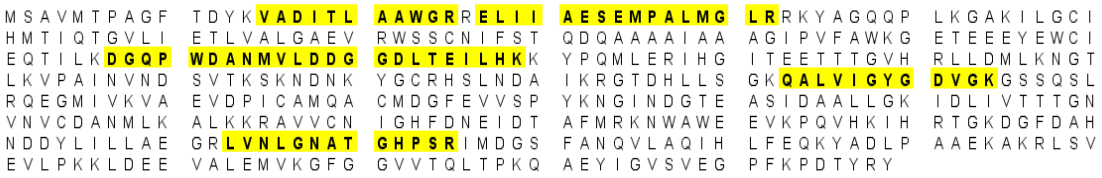
Appendix II

Protein Sequence Results (Scalfold)

P.aeruginosa clinical isolate control and NE treated proteins samples subjected to Liquid Chromatography - tandem mass spectrometry (LC/MS-MS). The obtained results were searched against Uniprot *Pseudomonas aeruginosa*_20100812 database. Scaffold software (version Scaffold_3.3.2, Proteome Software Inc., Portland, OR) was used to validate the results.

Adenosylhomocysteinase (51 KDa)

B7V419 (100%), 51,400.6 Da
Adenosylhomocysteinase OS=Pseudomonas aeruginosa (strain LESB58) GN=ahcY PE=3 SV=1
5 unique peptides, 5 unique spectra, 5 total spectra, 75/469 amino acids (16% coverage)



4- hydroxyphenylpyruvate dioxygenase (40 KDa)

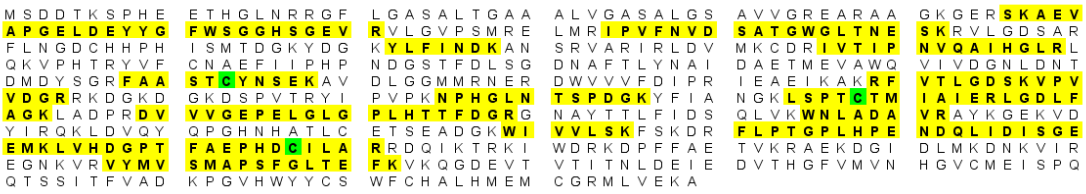
B7UY69 (100%), 39,930.2 Da
4-hydroxyphenylpyruvate dioxygenase OS=Pseudomonas aeruginosa (strain LESB58) GN=hpd PE=4 SV=1
17 unique peptides, 21 unique spectra, 22 total spectra, 192/357 amino acids (54% coverage)

Ferric Uptake Regulation Protein (15 kDa)

B7V1H6 (100%), 15,234.1 Da
Ferric uptake regulation protein OS=Pseudomonas aeruginosa (strain LESB58) GN=fur PE=4 SV=1
5 unique peptides, 5 unique spectra, 5 total spectra, 63/134 amino acids (47% coverage)

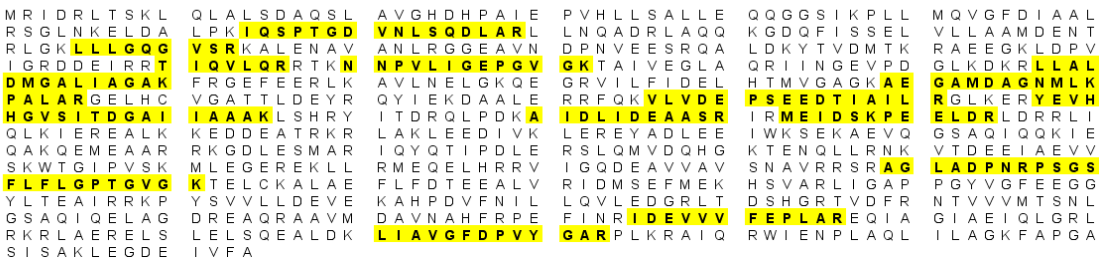
Nitrous-oxide reductase (71 KDa)

B7V129 (100%), 70,961.0 Da
Nitrous-oxide reductase OS=Pseudomonas aeruginosa (strain LESB58) GN=nosZ PE=4 SV=1
17 unique peptides, 19 unique spectra, 20 total spectra, 218/639 amino acids (34% coverage)



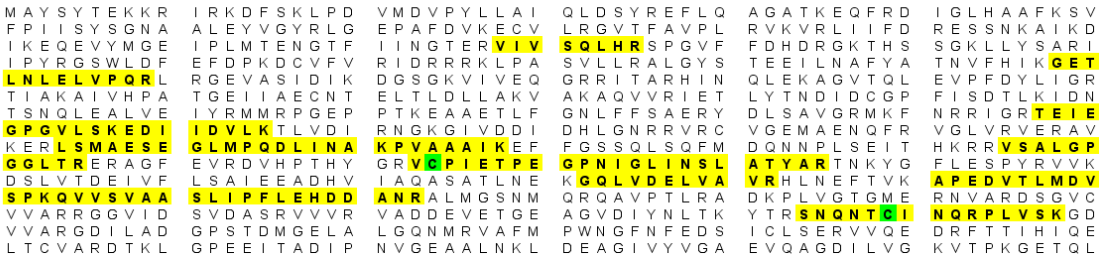
ClpB protein (95 KDa)

B7V085 (100%), 95,023.0 Da
ClpB protein OS=Pseudomonas aeruginosa (strain LESB58) GN=clpB PE=3 SV=1
13 unique peptides, 14 unique spectra, 14 total spectra, 182/854 amino acids (21% coverage)



DNA-directed RNA polymerase subunit beta (151 KDa)

A6UZI1 (100%), 150,869.8 Da
DNA-directed RNA polymerase subunit beta OS=Pseudomonas aeruginosa (strain PA7) GN=rpoB PE=3 SV=1
14 unique peptides, 15 unique spectra, 15 total spectra, 207/1357 amino acids (15% coverage)



Flagellin type (49 KDa)

B7UX97 (100%), 49,242.4 Da
Flagellin type B OS=Pseudomonas aeruginosa (strain LESB58) GN=fliC PE=4 SV=1
15 unique peptides, 16 unique spectra, 16 total spectra, 189/488 amino acids (39% coverage)

MALTVNTNIA SLNTQRNLNA SSNDLNTSLQ RLTTGYRINS AKDDAAGLQI SNRLSNQISG
LNVATRNAND GISLAQTAEG ALQQSTNILQ RIRDLALQSA NGSNSDADRA ALQKEVAAQQ
AELTRISDTT TFGGRKLLDG SFGTTSFQVG SNAYETIDIS LQNASASAIG SYQVGSNGAG
TVASVAGTAT ASGIASGTVN LVGGGQVKNI AIAAGDSAKA IAEKMDGAIP NLSARARTVF
TADVSGVTGG SLNFDVTVGS NTVSLAGVTS TQDLADQLNS NSSKLGITAS INDKGVLTIT
SATGENVKFG AQTGTATAGQ VAVKVQGSDG KFEAAAKNVV AAGTAATTTI VTGYVQLNSP
TAYSVSGTGT QASQVFGNAS AAQKSSVASV DISTADGAQN AIAVVDNALA AIDAQRADLG
AVQNRFKNTI DNLTNISENA TNARSRIKDT DFAAETAALS KNQVLQQAGT AILAQANQLP
QAVLSLLR

Aconitate hydratase (99 KDa)

B7UVG1 (100%), 99,149.1 Da
Aconitate hydratase 1 OS=Pseudomonas aeruginosa (strain LESB58) GN=acnA PE=4 SV=1
10 unique peptides, 11 unique spectra, 11 total spectra, 162/910 amino acids (18% coverage)

MPALDSLKTL RSLAVDGKTY HYYSLPEAAR TLGDLGKLPM SLKVLLENLL RWEDGSTVTG
DDLKALAGWL RERRSDREIQ YRPARVLMQD FTGVPAVVDL AAMRAAMAKA GGDPQKINPL
SPVDLVIDHS VMVDKFASES AFEQNVEIEM QRNGERYAFL RWGQNAFDNF SVVPPGTGIC
HQVNLEYLGR TVWTKDEDGR TYAFPDTLVG TDSHTTMING LGVLGWGVGG IEAEAAMLGQ
PVSMLIPEVI GFKLTGKLRE GITATDLVLT VTQMLRKKGV VGKFVEFYGD GLADLPLADR
ATIANMAPEY GATCGFFPVD EITLGYLRLS GRPESTVKLV EAYSKEQGLW REKGHEPVFT
DTLHLDMGEV EASLAGPKRP QDRVALQNVA SAFNEFLGLQ LHPSSTEEGR LLSEGGGGTA
VGANAAFGEI DYQHDGQTHR LKNGAVVIAA ITSCTNTSNP SVMMAAGLLA KKAVEKGLQR
KPWVKSSLAP GSKVVTDYFK AAGLTRYLDE LGFDLVGYGC TTCIGNSGPL LEPIEKAIQQ
ADLTVASVLS GNRNFEGRVH PLVKTNWLAS PPLVVAYALA GSVRINLSEE PLGTGKDGQP
VYLKDIWPSQ KEIAEAIQKV DTEMFHKEYA EVFAGDEKWQ AIQVPQSDTY EWQADSTYIQ
HPPFFEHIAE APPAIADVEQ ARVLAVLGDS VTTDHISPAG NIKADSPAGR YLREHGVEPK
DFNSYGSRRG NHEVMMRGTF ANIRIKNEML GGEEGGNTLY VPSGEQLAIY DAAMRYQEDG
TPLVIVAGKE YGTGSSRDWA AKGTNLLGVK AVIAESFERI HRSNLVGMGV LPLQFENGQD
RKSLKLTGKE VLNIRGLGGE LKPHMPLSVE VTREDGSQDS FKVLCRIDTL NEVEYFKAGG
ILHYVLRSML

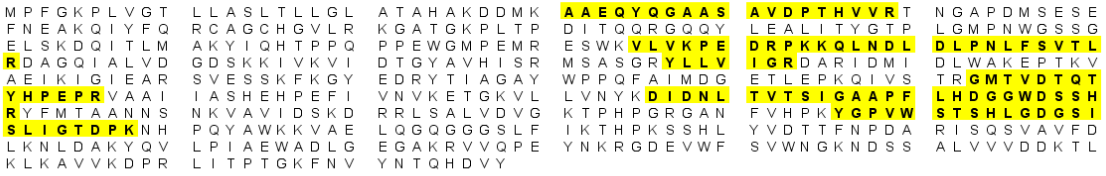
Outer membrane protein OprF (38 KDa)

A6V748 (100%), 37,639.0 Da
Outer membrane protein OprF OS=Pseudomonas aeruginosa (strain PA7) GN=oprF PE=3 SV=1
5 unique peptides, 5 unique spectra, 5 total spectra, 80/350 amino acids (23% coverage)

MKLKNTLGVV IGSLVAASAM NAFAQGQNSV EIEAFGKRYF TDSVRNMKNA DLYGGSIGYF
LTDDVELALS YGEYHDVRGT YETGNKKVHG NLTSLDAIYH FGTPGVGLRP YVSAGLAHQN
ITNINSDSQG RQQMTMANIG AGLKYYFTEN FFAKASLDGQ YGLEKRDNGH QGEWMAGLGV
GFNFGGSKAA PAPEPVADVC SDSDNDGVCD NVDKCPDTPA NVTVDANGCP AVAEVVRVQL
DVKFDFDKSK VKENSYADIK NLADFMKQYP STSTTVEGHT DSVGTDAYNQ KLSERRANAV
RDVLVNEYGV EGGRVNAVGY GESRPVADNA TAEGRAINRR VEAEVEAEAK

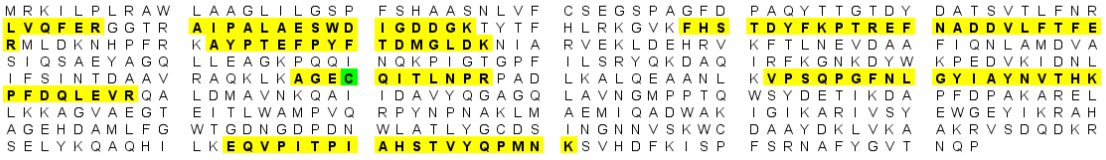
Nitrite reductase (63 KDa)

Q02TP7 (100%), 62,670.1 Da
Nitrite reductase OS=Pseudomonas aeruginosa (strain UCBPP-PA14) GN=nirS PE=4 SV=1
7 unique peptides, 8 unique spectra, 8 total spectra, 116/568 amino acids (20% coverage)



Putative binding protein component of ABC transport (60 KDa)

Q02GU1 (100%), 59,792.8 Da
Putative binding protein component of ABC transpor OS=Pseudomonas aeruginosa (strain UCBPP-PA14) GN=PA14_58390 PE=4 SV=1
4 unique peptides, 4 unique spectra, 4 total spectra, 119/533 amino acids (22% coverage)

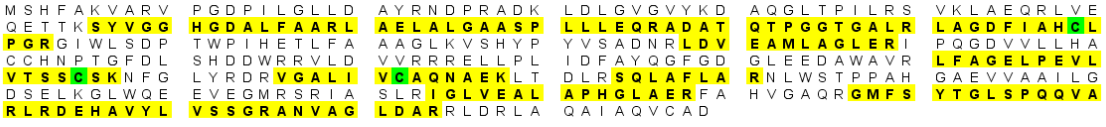


Glutamine synthetase (52 KDa)

A6VDN7 (100%), 51,946.0 Da
Glutamine synthetase OS=Pseudomonas aeruginosa (strain PA7) GN=glnA PE=3 SV=1
9 unique peptides, 11 unique spectra, 11 total spectra, 104/469 amino acids (22% coverage)

Aromatic-amino-acid aminotransferase (43 KDa)

P43336 (100%), 43,274.0 Da
Aromatic-amino-acid aminotransferase OS=Pseudomonas aeruginosa GN=phhC PE=3 SV=2
12 unique peptides, 13 unique spectra, 13 total spectra, 161/399 amino acids (40% coverage)



Putative universal stress protein (31 KDa)

B7VB90 (100%), 31,274.2 Da
Putative universal stress protein OS=Pseudomonas aeruginosa (strain LESB58) GN=PLES_35401 PE=4 SV=1
4 unique peptides, 4 unique spectra, 4 total spectra, 58/287 amino acids (20% coverage)

MQAIRSILVV IEPDQLEGLA LKRAQLIAGV TQSHLHLLVC EKRRDHSAAL NDLAQELREE
GYSVSTNQAW KDSLHQTIIA EQQAEGCGLI IKQHFPDNPL KKAILTPDDW KLLRFAPCPV
LMTKTARPWT GGKILAAVDV GNNDGEHRSL HAGIISHAYD IAGLAKATLH VISAHPSPML
SSADPTFQLS ETIEARYREA CRTFQAEYGF SDEQLHIEEG PADVLIPRTA QKLDAVVTVI
GTVARTGLSG ALIGNTAEVV LDTLESDVLV LKPDDIIAHL EELASKE

Alginate regulatory protein (35 kDa)

Q02EB0 (100%), 34,556.0 Da
Alginate regulatory protein AlgP OS=Pseudomonas aeruginosa (strain UCBPP-PA14) GN=algP PE=4 SV=1
3 unique peptides, 5 unique spectra, 5 total spectra, 38/352 amino acids (11% coverage)

MSANKKPVTT PLHLLQQLSH SLVEHLEGAC KQALVDSEKL LAKLEKQRGK AQEKLHKART
KLQDAAKAGK TKAQAKARET ISDLEEALDT LKARQADTRT YIVGLKRDVQ ESLKLAQGVG
KVKEAAGKAL ESRKAKPATK PAAKAAAKPA MKTVAAKPAA KPAAKPAAKP AAKPAAKTAA
AKPAAKPAAK PVAKPAAKPA AKTAAAKPAA KPAAKPVAKP AAKPAAKTAA AKPAAKPAVK
PVAKPAAKPA AKTAAAKPAA KPAAKPVAKP AAKPVAKPAA AKPAAKPAAK PAAKPVAKPA
AKPVAAKPAA AKPATAPAAK PAATPSAPAA ASSAASATPA AGSNGAAPTS AS

Chitinase (53 KDa)

Q02M97 (100%), 53,127.3 Da
Chitinase OS=Pseudomonas aeruginosa (strain UCBPP-PA14) GN=chiC PE=3 SV=1
19 unique peptides, 27 unique spectra, 32 total spectra, 192/483 amino acids (40% coverage)

MIRIDFSQLH QAREDAAAAM PSIAGKKILM GFWHNWPAGA ADGYQQGSFA NIALEDVPSE
YNVVAVAFMK GRGIPTFQPY NLSDAEFRRQ VGVLNAQGRA VLISLGGADA HIELHAGQEQ
ALAAEIVRLV ETYGFDGLDI DLEQSAIDLA DNQRVLPAAL KLVREHYAGQ GKHFIVSMAP
EFPYLHKNGK YVPYLQALEG VYDFIAPQYY NQGGDGLWVQ EANGGKGAWI AQNNDAMKED
FLYYLTESLA TGSRDFVRIP AQRLAIGLPS NVDAAATGYV IDPAAVSNAF RRLEAAGHAI
KGLMTWSVNW DDGLNKRGER YNWEFRKRYA SLIHDDEGGD QRPAPPQGLR LLERGETSLV
LAWNASSGQR PIDYYSLYRD GAMVGQSAAL GSTDSGLTAD TRYSYFVTAT DTQGNQSLPS
EGLEVSTSGG AVDPQFPQWR ENQAYRVDDG VTYEGLRYLC LQAHTSNSGW TPPVAFTLWR
PLR

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